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GASTRIC GLANDULAR TUMOURS PROVIDED WITH EXCRETORY DUCTS, AND CRITICISM OF THE THEORY OF THE TUMOURS ARISING IN HETEROTOPIC PANCREAS

Observations on the occurrence of atypical glands in the stomach

By

OSMO JÄRVI and PEKKA LAURÉN[1]

Received 30 xi 63

Some workers contend that the structure of gastric tumours merits no major attention as the structure may vary so much in the different parts of the tumour that one type of histological structure may be transformed into another (*Stout* 1943, 1953, *Ackerman & del Regato* 1962) The present authors however, hold that the structure also of gastric tumours warrants at least some conclusions concerning the origin of the tumours, and that the establishment of the type of tumour structure on which a classification may be based is of both theoretical and practical importance Similar views were advanced e g by *Borrmann* (1926) and *Willis* (1960) In their study from 1951 on the histological structure of gastric cancer, the authors found that the tumours derived from metaplasias of the intestinal epithelium of the stomach in at least 50 per cent of the cases This observation was later confirmed by several other workers (*Mulligan & Rember* 1954 *Morson* 1955 *Wattenberg* 1959 and *Planteydt & Willighagen* 1960) When the authors continued their studies in order to classify histogenetically all epithelial tumours of the stomach two tumours the histological structure of which differed distinctly from other tumours were separated from the material comprising more than one thousand investigated cases and classified into a special group In both cases it was possible to distinguish in the tumour tissue a system of ducts opening into the mucosa as well as specific secretory cells forming glandular acini[2]

CASE REPORTS

Case 1 (6015 59) was a 44 year old wife of a worker with several years history of gastric trouble She felt acute pain in the right epigastrium and in the side after

[1] The work of P Laurén was supported by the *Damon Runyon Memorial Fund*

The authors thank Professor *E Vuori* and Dr *R Jäfs* for providing the clinical reports on patients treated at Loimaa District Hospital and the Pori Municipal Hospital

rich meals and her complaint had been treated as anacid dyspepsia Gallstones were found at examination on March 2 1959 at the Pori Municipal Hospital and an operation was performed at which a single large stone was found in the gallbladder Four months after the operation the patient began to complain again of pain similar to her preoperative pain She had no appetite and lost 5 kg in weight in the course of the spring At examination a mass the size of a small egg was palpable through the abdominal wall and a large round filling defect was revealed by X ray of the stomach The gastric juice was deficient in acid total acidity being 7 At operation on August 7 1959 (Dr *Idfs*) an egg sized round mobile tumour covered by an intact smooth mucosa was found in the stomach at the level of the angulus The mucosa however was slightly irregular in some places The tumour was removed The patient made a good postoperative recovery but gastric pain recurred one month later The patient was referred to the Surgical Clinic University of Turku where gastric resection was undertaken on October 26 1959 No new tumour tissue was found at the operation nor was such tissue demonstrable by microscopic examination of the samples The patient last attended for a follow up on February 14 1961 She complained of back pain and micturition difficulties but no longer of abdominal pain Nothing of note was found at the physical examination Sugar metabolism tests were not carried out prior to the removal of the tumour Nothing in the anamnesis suggested hypoglycaemia No sugar was found in the urine In a letter of September 4 1963 the patient reported that she was in good health

Case 2 (3920/61) was a 53 year old fitter who after a few weeks history of pain of gastric ulcer type on April 30 1961 had an attack of severe upper abdominal pain lasting for about 1 hour The patient was admitted to the Loimaa District Hospital where an operation was performed (Dr *Haitanen*) A perforated ulcer admitting a finger was observed close to the cardia in the lesser curvature and was closed with sutures Microscopic examination of the excised specimen displayed carcinomatous tissue Hence operation was repeated on May 15 at which tumour tissue was found below the cardia In addition numerous enlarged lymph nodes were observed in the region between the stomach and the spleen The case was considered inoperable and the operation wound was closed At follow up examinations the patient was still in poor condition and unfit for work He had lost some weight recently X ray examination on October 5 1961 disclosed a distinct but fairly small sized ulcer niche in the body of the stomach this was interpreted as a well demarcated cancer The rest of the stomach looked normal The patient was given several post operative courses of radiotherapy and from March 1962 was placed on 100 mg of Sendoxan per day He was admitted to the Loimaa District Hospital with pneumonia on January 29 1962 and again to the Surgical Clinic University of Turku in October 1962 The case history included no symptoms associated with disorders of sugar metabolism The blood glucose level on October 30–November 7 1962 was 85 110 mg per cent The Wohlgemuth test gave 32 Wohlgemuth Units 45°/15 for urine and 4 W U 45°/15 for blood Titz lipase was 0 40 T units The glucose tolerance test was normal Creatinine in the blood was 0 6 mg per cent Alkaline phosphatase in the serum was 6 8 B L units acid phosphatase 0 46 mg per cent 17 ketosteroid determined in urine 15 5 mg/day X ray examination on October 30 1962 revealed a crater of medium size in the posterior wall in the superior part of the body of the stomach There was no characteristic walling off around the crater and it was not considered typical of malignant growth X ray of the thorax revealed a considerable quantity of fluid in the left pleural sinus The interlobar pleural boundaries were clearly visualized and paracardially to the left in the region of the inferior lobe dense infiltration which was interpreted as pneumonic was seen to extend laterally down from the hilus At examination on November 28 the X ray finding in the stomach was unchanged The patient however was in poor condition and died on December 8 1962 Unfortunately no autopsy was performed

Histological Studies

In case 1 the microscopic examination was performed on a specimen taken at the operation on August 7 1959 and in case 2 on a specimen taken at the operation on April 30 1961 The tumours differed so little from one another that a general description of their structure will suffice



Figs 1 and 2

Fig 1 Case 1 azan staining General view of the tumour A richly ramified duct opens into the mucosa at the right edge of the picture A part of the terminal acini are located in the basal portion of the mucosa There are large lobes of acinar tumour tissue in the submucosa × 25

Fig 2 Case 1 azan staining red filter The epithelium of the ducts has intestinal columnar epithelium with brush border (*e g* high epithelium on the right) *side by side* with neutral mucus secreting gastric surface epithelium (cells staining *dark grey apically*) Goblet cells (dark isolated cells) are more numerous in the area of gastric than of intest[illegible]
buds adjoin the d[illegible]
[illegible] usly also *solid* [illegible]
darker grey in sev[illegible]

1[illegible]

Figs 3 and 4

Fig 3 Case 1 PAS technique green filter Demilune shaped mucoserous terminal acini or more complex adenomere structures adjoin the mucous tubules composed of neutral mucus containing cells The course of the basement membrane is accentuated with Indian ink in the middle of the picture the approximate contours of the intercellular secretory capillaries are accentuated similarly × 350

Fig 4 Case 1, PAS technique The epithelium of gastric type in the ducts stains magenta with PAS Numerous fine secretion granules which have stained more faintly are to be seen in the cells of the terminal acinar conglomerates or long straight cell cords both often provided with secretory capillaries × 200



F gs 5 and 6

F g 5 Ca e 1 Mas on Hamperl s techn que Some enterochr maff ne cells sta nable wi h Masson s technique are encountered in the term nal acinar part of the tumour × 350

F g 6 Ca e 2 haematoxyl n van C eson sta ning A general v ew of the tumour To the right two of the ducts opens into the mucosa The mucosa s of normal l o l stru ture In c ntrast the submucosa shows numerous complexes of ac nar ti sue of the tumour adjoining the ducts × 25



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Fig. 3. Case 1, PAS technique, green filter Demilune-shaped mucoserous terminal acini or more complex adenomere structures adjoin the mucous tubules composed of neutral mucus-containing cells. The course of the basement membrane is accentuated with Indian ink in the middle of the picture; the approximate contours of the intercellular secretory capillaries are accentuated similarly. × 350.

Fig. 4. Case 1, PAS technique. The epithelium of gastric type in the ducts stains magenta with PAS. Numerous fine secretion granules which have stained more faintly are to be seen in the cells of the terminal acinar conglomerates or long straight cell cords, both often provided with secretory capillaries × 200.



Figs 5 and 6

Fig 5 Case 1 Masson Hamperl's technique Some enterochromaffine cells stainable with Masson's technique are encountered in the terminal acinar part of the tumour × 350

Fig 6 Case 2 haematoxylin van Gieson staining A general view of the tumour *To the right two of the ducts opens into the mucosa* The mucosa is of normal structure *In contrast the submucosa shows* numerous complexes of acinar tissue of the tumour adjoining the ducts × 25



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furthermore tubulus like terminal formations or narrow solid cell cords and rows which have no counterpart in the normal gland structure (Figs 2 and 4) The last mentioned may constitute extensive uniform tumour lobes with only thin septa of connective tissue between the cords (Figs 1 4 and 5)

It was possible to distinguish in the ducts primarily two types of epithelium one corresponding to gastric surface epithelium and one corresponding to intestinal epithelium The cells of the former type contained neutral mucus and stained magenta red with the periodic acid Schiff technique (PAS) they stained fairly intensely with Best s carmine but remained practically unstained with mucicarmine and alcian blue The intestinal epithelium again consisted of columnar cells with a brush border and of goblet cells irregulary spaced between the former cells The goblet cells contained acid mucus and stained bright red with the PAS technique medium blue with Heidenhain s azan trichrome they stained well with mucicarmine and alcian blue but were generally negative to Best s carmine Goblet cells could also occur in the gastric surface type epithelium of the ducts (Fig 2) The secretion of mucinous glands and tubules stained like the neutral mucus in antral and duodenal glandular cells It was negative with mucicarmine and alcian blue but stained rather heavily with Best s carmine It differed from the mucus of gastric surface epithelium by the bright red colour with PAS faint blue tint with azan stain The cells in the ducts however were not completely regular The nuclei could be irregularly grouped and variations in nuclear size were also observed Mitoses were also encountered occasionally

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The cellular structure was indicative of malignancy in both cases In addition to architectural disorders of form and composition of the ducts



Fig 7

Case 2 azan staining, red filter Side branches adjoin the main ducts of the tumour and are connected with extensive terminal acinar complexes Numerous blue staining secretion granules are to be seen in the cells of acinar tissue and partly in the duct cells as well × 300

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Anatomy, University of Turku, and comparison of the slides showed a similarity between the ductal structures seen in the cases reviewed by the present authors and those of the accessory pancreas. In addition, epithelium containing goblet cells and epithelium in which the cells contain neutral mucus resembling gastric surface epithelium, and, furthermore, pseudopyloric glands connected with the duct have been described together with epithelium secreting acid mucus in the ducts of normal pancreas (*Zimmermann* 1927, *Gruber* 1929, *Feyrter* 1931, *McMinn & Kugler* 1961). The acinar cells encountered in the tumours reported here, however, are poorly compatible with a pancreatic origin. The secretion of the cells differs clearly from the secretion of pancreatic exocrine cells and is mucoserous in quality. Ergastoplasm of the type encountered in pancreas cells was not observed and the centroacinar cells of the neck parts which are characteristic of the pancreas were absent. On the other hand, the attention was attracted by the structural similarity of the cell cords encountered, especially in case 1 to those seen in tumours described as insulomas of the pancreas. However, mucinous or mucoserous secretion has not been reported in connection with insulom[illegible]

corresponding secretion granules in the tumour cells nor basal and supranuclear ergastoplasm nor intercellular secretory capillaries. Hence the similarity of gastric duct tumours reported in this paper and malignant insuloma was confined to the rough architecture, the tumours being completely different in their internal structure.

Relation of the Tumours to Glandular Abnormalities of the Stomach

The following case may serve to throw additional light on the origin of the tumours.

Case 3 (made available to the *authors by Dr H Arko*, Central Hospital of Kuopio) was a newborn male baby. A pea sized, pale, flat intumescence was observed in the antral part of the stomach at *the site of* the greater curvature. Microscopy (Fig. 8) showed the intumescen[illegible]
The d[illegible]
type[illegible]

[illegible] these cells contained granules which [illegible] with azan partly blue, partly *red*. *The* granules, however, were fairly large and resembled zymogen granules.

In this case the groups of cells adjoining the ducts resembled islets of Langerhans (Fig. 9), particularly the cell groups occurring in connection with pancreatic ducts, earlier reported by *Feyrter* (1939) and *Baumann* (1939) as 'insular duct organ'. A corresponding tumorous

and acinar tissue and occasional lack of secretory activity in the cells, nuclear irregularities were seen The vesicular nuclei had a rough though loose chromatin network and the nucleoli were distinctly enlarged Multinuclear giant cells with up to 24 nuclei were encountered occasionally Here and there some mitoses were seen In case 2 the tumour tissue grew distinctly inside the lymph vessels as well and penetrated the muscularis mucosae in many places from the submucosa, invading the basal parts of the otherwise well-preserved mucosa In case 1 the tumour appeared to be better demarcated and it was not possible to demonstrate any growth in the lymph vessels The cellular architecture, however, was more disordered in the acinar tissue in case 1 than in case 2

To summarize the microscopic findings, both cases involved a highly differentiated tumour with exocrine secretion located principally in the submucosal layer of the stomach and draining into the mucosa through several ducts No proliferation of the smooth muscle layer was seen to be associated with the tumour The ductal parts of the tumour contained both gastric surface epithelum and intestinal epithelium and to a small extent also mucinous tubules of antral type Terminal secretory acini which may constitute extensive irregular complexes or cords were associated with the ducts without intercalation of the neck parts The acinar cells belonged to the group of mucoserous salivary gland cells with regard to their structure and secretion The tumours were malignant in their histological structure, but obviously could have grown slowly and remained fairly well demarcated (case 1)

DISCUSSION

Differential Diagnosis from the Tumours of Heterotopic Pancreas

Comparison of the present cases with earlier literature may primarily suggest that they belong to the group of heterotopic (ectopic) tumours

The best-known heterotopias in the stomach are the presence of intestinal epithelium in the gastric mucosa, the invasion of the submucosa by antral type (pseudopyloric) glands, and the occurrence of pancreatic tissue in different parts of the stomach wall Cases in which pancreatic duct tissue is encountered without acinar tissue have also been described as adenomyomas or rudimentary accessory pancreases (*Feyrter* 1931) The occurrence of islets of Langerhans is also variable they are frequently noted in exocrine gland tissue occasionally they are absent, but cases have also been reported presenting ducts and islets only and no exocrine cells No pancreatic tissue of normal structure was encountered in the present cases and all "heterotopic tissue was tumorous However, the ducts of the tumour were fairly similar to ducts occurring in accessory pancreases Corresponding stainings were performed in the case of pancreas accessorium of the stomach, published in 1959 by *Rintala* from the Department of Pathological

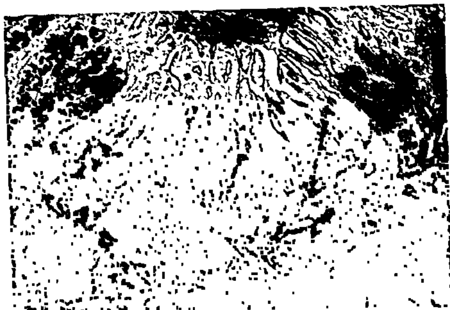


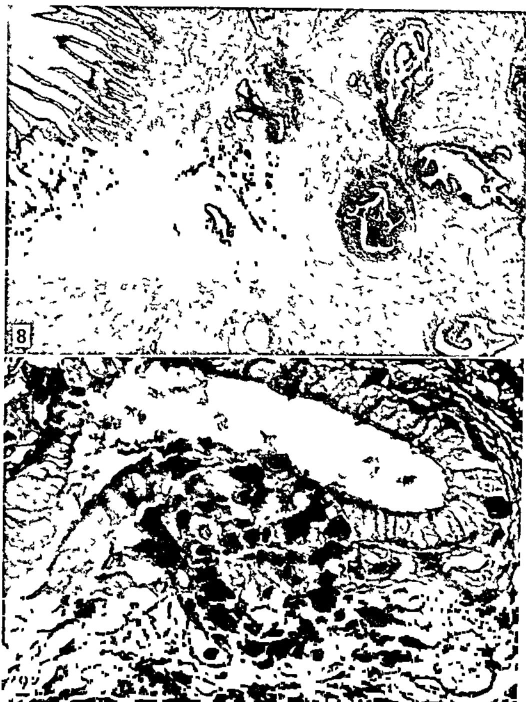
Fig 10

Case 4 PAS technique A gland group of peculiar type with serous and mucoserous cells, between normal antral glands in an ulcer stomach The secretion granules stain moderately with PAS To the right at the edge of the picture, also intestinal metaplasia in the epithelium × 40

growth in the pancreas was described as "nesidioblastoma" by *Laidlaw* (1938) and *Frantz* (1959) On the other hand, the cell groups might also have been as yet completely undifferentiated exocrine pancreatic tissue or still undeveloped initial tissue comparable with the acinar tissue of the tumours described in the foregoing No doubt the tumours considered here might be assumed to derive from congenital development disturbances of this type

It has proved possible in experiments to make the gastric surface epithelium penetrate the muscularis mucosae into the submucosa, *e g* by placing carcinogens in the stomach wall (*Howes & de Oliveira* 1948, *Stewart* 1953) *Jarvi & Keyrilainen* (1955) and *Järvi* (1962) reported that neck epithelium as well as antral (pseudopyloric) glands were connected with the cavities thus formed On the other hand, cells with *mucoserous secretion have been encountered in the antral and duodenal* glands of many different animals (*Schwalbe* 1872, *Bensley* 1903, *Villemin* 1920, *Tschassownikow* 1926, *Tehver* 1929, *Burkl* 1950) This finding was confirmed by *Jarvi* in mice[1] Also in man glands may penetrate the submucosa or the deeper layers secondarily as so-called pseudopyloric heterotopic glands and, in addition, surface and neck epithelium in cases of giant hypertrophic gastritis (cf *Jarvi* 1962)

[1] Not published



Figs 8 and 9

Fig 8 Case 3 haematoxylin mucicarmine staining The mucosa to the left is of antral structure The muscle layer displays ducts of varying morphology consisting partly of pale higher gastric surface epithelium partly of darker intestinal epithelium There are in addition minor ducts in which the cells contain acid mucus and adjacent to them somewhat vaguely demarcated cell groups × 40

Fig 9 Case 3 azan staining green filter A cell group reminiscent of islets of Langerhans adjoins the duct The cells contain granules staining partly blue partly red with azan × 350



Figs 11 and 12

Fig 11 Case 5, haematoxylin van Gieson stain Mixed seromucinous glands in the cardiac mucosa The cells of the mucinous tubules contain neutral staining *mucus, the demilunes and the acini are "mucoserous" The surface epithelium* is partly intestinal type × 30

Fig 12 A detail from Fig 11 showing the mucoserous demilunes and acini adjoining the mucinous tubules The contours of the secretory capillaries in the former are accentuated with ink × 300

Furthermore, cells which differ from those described in textbooks and handbooks may be demonstrated in human gastric mucosa Serous or mucoserous cells of this type have in general been assumed probably to represent only a certain secretory phase of antral glandular cells and they have, to a certain degree, escaped attention The only exceptions are reported by *Hintzsche* (1939) and *Burkl* (1950), who described heterotopic serous acini and semilunes in connection with duodenal and antral glands in the stomach In spite of the differing architecture of the cell grouping they suggested that acini might represent pancreatic tissue *King & MacCallum* (1934) found one pancreatic acinus adjoining duodenal glands in a case with accessory pancreas in the pylorus Here two examples are described of gland groups of divergent glandular structure located in the gastric mucosa

Case 4 (made available to the authors by Dr *Virkkunen* Central Hospital of Northern Karelia) was a 56 year-old railway ganger The patient had suffered from gastric pain for 4 years X-ray revealed a gastric ulcer A large gastric ulcer close to the cardia was found at operation A specimen taken from the edge of the ulcer showed in the cardiac mucosa a gland conglomerate which differed from the environment (Fig 10) The glands were acinotubular Secretory capillaries were seen between the cells In their apical part the cells contained well preserved secretory granules which distinguished them from the antral gland cells the secretion of which was reticular The secretion in the cells was often divided into two groups the supranuclear Golgi apparatus group and the group located at the cell apex The granules stained, sometimes red with azan sometimes purple or blue With the PAS technique however they all gave a pale red reaction Mucicarmine and alcian blue also produced a faint staining The cells are consequently comparable with the serous and mucoserous cells of the salivary glands No differentiated ducts were associated with the glands which drained directly into the foveolae of the mucosa

Case 5 (made available to the authors by Dr *Slatis*, Central Hospital of Vaasa) was a 41 year old policeman presenting a year s history of gradually increasing pain in the abdominal region and occasional vomiting X ray examination disclosed an extensive constriction in the body of the stomach, a mass was palpated also in the epigastrium At operation a constricting tumour was found in the body of the stomach Microscopic examination revealed a scirrhous carcinoma The gastric surface epithelium was displaced by intestinal epithelium over wide areas

The second specimen in which no tumour tissue was disclosed taken from a site close to the cardia revealed an atypical gland conglomerate about 1 5 mm in diameter in the basal part of the mucosa The glandular tubules had lost their usual vertical course in the mucosa and ramified freely in the environment (Figs 11 and 12) Demilune-shaped terminal cell groups or fully developed acini were found in many places in association with the mucinous tubules The mucinous cells of the tubules corresponded in structure and in the stainability of their secretion to cells of the cardiac (as well as antral) mucosal glands The mucus stained well with Bests's carmine and bright red with PAS, it did not take mucicarmine or alcian blue and stained faintly blue by the azan technique The acinar cells however had a conical shape the apex pointing to the narrow central lumen the cells were separated from each other by secretory capillaries The nuclei were round and not flat as is often seen in mucinous cells a weakly stainable ergastoplasmic lamellar system was found both basally and in the supranuclear zone The cell cytoplasm contained well preserved secretory granules These stained with various brown colours in haematoxylin van Gieson stain negative with Best s carmine and moderately positive by the PAS technique

In these cases, accordingly, foci of acinotubular glands clearly deviating from normal antral, cardiac or body glands occurred in the cardiac mucosa Because of the neutral character of the mucus of the

any cancerous or adenomatous growth originating from the gastric tubules These tubules are not in connection with this submucous growth The granular mass consisting originally of the misplaced pancreatic tissue is the starting point of a true tumor formation characterized by irregular alveoli usually lined but sometimes filled with epithelial cells of the same general type as those of the acini corresponding to the pancreatic tissue, but more polymorphous in character" Taking into consideration that two pathologists had regarded the case as a pyloric carcinoma without any mention of pancreatic heterotopia and that *Welch* stated that the granular mass (suggested heterotopic pancreas tissue) had displayed certain deviations from the normal type, and stressed the difference of the tissue from any tumorous growth originating from the gastric tubules the authors consider it probable that the tumour involved resembles those reported here with the maturity of the tumour tissue varying in different places but in which real normal pancreas tissue was hardly demonstrable anywhere

A case resembling the one reported by *Branham* was described by *Nicholson* (1923) In a 57 year-old man who died in an accident, a firm oval tumour measuring 4 cm in diameter was found on the greater curvature of the stomach extending from the mucous membrane beneath the peritoneal covering *Nicholson* writes "On comparing it with normal pancreas and with carcinomata of this organ there can be no question that it is composed entirely of carcinomatous pancreatic epithelium" Since there was no new growth in the pancreas the only remaining possibility is that it was a matter of a malignant accessory pancreas

Askanazy (1923) reported a solid carcinoma likewise of submucosal origin The tumour was characterized by slow progression and slight clinical symptoms Also the gallbladder and the cystic duct were absent On the basis of these arguments the author assumed that the tumour started from an accessory pancreas but he was unable to detect it

Further *Borrmann* (1926) mentioned briefly the finding of a solid carcinoma which might have derived from pancreatic tissue but failed to display any definite signs indicative of an accessory pancreas.

In 1937 *May* described a cystic, degenerated solid carcinoma with a structural resemblance to islets of Langerhans in a patient in whom neither glycosuria nor clinical symptoms of diabetes were manifest but who at one examination was found to have hyperglycaemia of 240 mg per cent In addition an ulcer was observed close to the tumour On the basis of the last mentioned observation *May's* tumour was interpreted by *Ellison* (1962) as a pancreatic insuloma of delta cell type in the stomach in spite of *May's* statement that the bottom of the ulcer was already macroscopically infiltrated by tumour tissue

In a table of their material of accessory pancreases *Barbosa et al* (1946) listed a case in which the pancreas was located directly below a carcinomatous ulcer Malignant change of accessory pancreas was suggested but no description of the tumour was given

A large ulcera[illegible]
as a benign non[illegible]
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tumour schwar[illegible]
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In 1960 S[illegible] a[illegible] functionally quiescent tumours in the pylorus interpreting these as insulomas but failed to give any information about the tumour structure or the relationship to the occurrence if any, of heterotopic pancreatic tissue

In 1962 *Horangi* & *Buday* established the [illegible]

[illegible] established [illegible]
described by *Branham*

After the manuscript was completed the authors found the paper by *Goldfarb et al* (1963) in which a ductal mucus producing pyloric adenocarcinoma arising in a fully developed heterotopic pancreas was adequately described

mucinous cells, the glands in the latter case resembled primarily the duodenal seromucinous glands occurring in some mammals No special duct system was present in these cases

The atypical glands found in the two cases described here closely resemble the glands occurring in the cases reported by *Hintzsche* and *Burkl* The writers, however, are not inclined to follow these authors in interpreting the serous cells as pancreatic *Burkl* points out some divergency in structure of part of the cells and the strange way in which the serous cell groups adjoin the mucinous tubules, which is comparable rather with a seromucous salivary gland than with pancreas It may be mentioned that *Jarvi & Meurman* (1964) had observed pancreatic acini associated with metaplasia of gastric surface epithelium in the gall bladder mucosa, but the cell structure and grouping of the cells into acini seen in this case differed markedly from the cases described above It is interesting to note, however, that *Burkl* was able to collect from among 47 pathological gastric specimens a total of 8 cases presenting these atypical glands, he suggested that the glands had arisen as acquired metaplasia resulting from moderate gastritis Further, he found some intercalated ducts adjoining these glands, which rather strengthens the comparison with the tumours provided with a ductular system reported in this paper, though it can hardly be denied that there is a difference as regards the extent to which the ducts in both categories have developed

Thus, considering the above cases it seems possible that mucoserous acinar tumours with ducts in the gastric wall may arise in the following ways (1) on the basis of a congenital developmental disorder involving some salivary type gland or possibly pancreatic tissue (2) on account of some irritation of the mucosa and wall of the stomach from which glandular metaplasia might also ensue In this phase of the investigation, it remains an open question whether one or the other of these alternatives apply

Review of the Literature on the Solitary Gastric Tumours Suggested to Have Arisen in Heterotopic Pancreas

The connection in one way or another of the tumours described above with accessory pancreas is supported by reports in the literature which however do not take other alternatives into consideration In 11 cases the tumour is assumed to originate from pancreatic tissue in the stomach but the reasons for such an assumption are vague in all of the cases

In the case reported by *Pforringer* in 1904 the solid carcinomatous tissue seemed to be of submucosal origin There was a great deal of accessory pancreatic tissue in the duodenum but the tumour was not found to be connected with it at any site

In 1908 *Branham* reported a 20 year old patient with a tumour in the pylorus The tumour was first diagnosed *as a polyric carcinoma but Prof W H Welch* interpreted it as a malignant adenoma derived from an accessory pancreas As *Welch's* statement allows of several interpretations the essential passage is cited here In the submucous coat is a granular mass presenting the structural features of the pancreas with certain deviations from the normal type There are spherical and oval acini lined or filled with cubical or cylindrical granular epithelial cells these acini corresponding in shape and general appearance to those of the pancreas and unlike

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In 1962 Horanyi & Buday established the diagnosis of a heterotopic adenomyosis which had become malignant at the *site of a gastroenteroanastomosis* 40 years after gastric intervention

Accordingly reports of tumours in the stomach

manuscr

Review of the Literature on the Solitary Intestinal Tumours Suggested to Have Arisen in Heterotopic Pancreas

A number of tumours similar to those described in the preceding paragraph have been found in the lower parts of the gastrointestinal tract

A cystic degenerated carcinoma in the duodenum the structure of which resembled pancreatic tissue was reported by *Bookman* (1932), but the article gives little in formation about the tumour

In 1943 *Duff et al* described a duodenal adenocarcinoma which on the basis of the deep location in the duodenal wall and of the microscopical structure of the tumour they thought to be comparable with the pancreatic ducts regarded as deriving from accessory pancreas

Further *Holman et al* reported in 1943 an apparently hormonally quiescent case presenting a tumour structurally of the islet type and located in the duodenum No pancreatic acinar or ductal tissue were seen

In a case reported by *Brown et al* (1944) a stenosing tumour of the ascending portion of the duodenum was resected Microscopic studies of the mass showed a new growth arising from pancreatic tissue Since the pancreas was normal it was concluded that the tumour originated from accessory pancreatic tissue in the duo denal wall (which tissue apparently could not be demonstrated) The character of the tumour strongly suggested that the aberrant tissue was ductal in origin

One case is included in the table published by *Barbosa et al* in which an accessory pancreas is overlying a carcinomatous duodenal ulcer at the outlet of the common bile duct Malignant change of accessory pancreas was suspected

Silver & Lubliner (1948) in connection with their report of 104 pancreatic tumours briefly mention a 80 year old women with the clinical diagnosis of gastric ma lignancy At autopsy an adenocarcinoma was found which was reported as having arisen in an accessory pancreas of the duodenum The authors do not consider the case as islet cell tumour

Oberhelman et al (1958) reported a patient who repeatedly had gastric ulcers A non insulin producing islet type tumour in the duodenum with suspicion of lymph node metastasis to the region of the head of the pancreas was found Apparently normal pancreatic tissue was absent in the duodenum but the figure of the lymph node shows in addition to islet type cords distinct acinar tissue though whether tumorous or not cannot be evaluated In the discussion the authors mentioned one more case of duodenal non insulin producing islet cell tumour associated with clinical steatorrhea syndrome

For completeness it may be added that the 7 cases by *Scagliosi* (1913) described as duodenal carcinomas or beginning carcinomas arising in connection with the accessory pancreas presented evidently no true tumour as *Feyrter* (1931) had al ready stated In all of these 7 cases the suggested tumour of microscopical size re presented an additional finding at autopsy

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Non-Insular Tumour Cases Suggested to have Arisen in Heterotopic Pancreas Outside the Stomach and Intestine

The authors have found only three cases of this category described in the liter ature *Silver & Lubliner* reported an autopsied case A 53 year old man had an — f the accessory pancreas in the porta hepatis with numerous
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porta as well as the cystic and hepatic duct were

The study confirms earlier observations that these ruptures occur independent of age and pathologic changes of the aorta. Middle-aged men form the largest group, probably because they most often expose themselves to serious traffic accidents. In contrast to earlier observations the material contains more motorists than pedestrians. This is probably because most accidents happened on country roads where, as civilization proceeds, pedestrians are a vanishing race.

Traumatic rupture of the aorta is usually accompanied by fatal intrathoracic haemorrhage. The survival time is only in a few instances long enough to permit surgical treatment. In a number of cases this injury is the single serious lesion in cases of traffic accidents. There are therefore ample reasons to analyse, as far as possible, the mechanical forces acting in these instances, in order to find adequate counter measures. Such analysis is the subject of another study (*Lundevall* 1964).

SUMMARY

Twenty-one cases of fatal traumatic ruptures of aorta are reported. All cases except one were traffic accidents. Middle-aged men were the largest group of the injured. The ruptures were predominantly located at the isthmus of aorta. In 16 cases only one rupture occurred, in 5 cases there were 2 or more ruptures. Severe intrathoracic haemorrhage was present in all cases and probably represented the single cause of death in 5 cases.

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Oppenheim did not state at which rate the pressures were applied, but it is inferred from his remarks that this was rather slow

Klotz & Simpson (1932) found in similar experiments that aorta from young persons resisted an internal pressure of 1000 mm Hg without rupturing, but they nevertheless assumed that it is the sudden rise of the blood pressure which ruptures the aorta during accidents Even *Kleinsasser* (1943), *Strassmann* (1947) and *Tannenbaum & Ferguson* (1948) were of the opinion that a rise in blood pressure is an important factor for the occurrence of these ruptures

No experiments have been reported in which the blood pressure in the thoracic portion of aorta has been measured during impacts to the body

A number of authors have stressed the importance of aorta's topography in order to explain the mechanism of rupture *Letterer* (1924) thought that in cases where the individual fell on his feet or buttocks from a height, the rupture was caused by the heart s downward traction on the aorta

Shennan (1929) held the opinion that the relative fixation of the aorta to its surroundings at isthmus had a "hinge" effect, causing rupture at this point by sudden movements of the chest, and *Kleinsasser* (1943) sustained this opinion

Hass (1944) more generally stated that any rapid relative displacement of two adjacent parts of the body causes a stress at the junction, and that this is the basis for the mechanism of aortic rupture *Tannenbaum & Ferguson* (1948) agreed to some extent, but said that some cases of traumatic rupture of the aorta could not be explained by Hass' theory, as for instance thoses cases in which rupture is caused by flying pieces of wood or stone hitting the chest

Marshall (1938) pointed out that when the body is decelerated during forward motion the hilus of the left lung will exert a traction on the parietal pleura covering the aorta By this mechanism aorta is probably kinked in such a way that rupture occurs

Cammack et al (1959) found that anterio-posterior compression of the chest will cause a dislocation of the heart to the left, and a torsion of the aorta with a maximal stress on its wall near the base of the heart

Zehnder (1960) deduced from theoretical considerations that the main factor for different traumata is a hyperflexion of the aortic arch, with a predominant stress on the isthmus region

To summarize, earlier authors agree that *traumatic rupture occurs* in connection with a considerable and sudden change of velocity of the body The direction of the velocity change may vary from one case to another and is often but not always accompanied by a contusion of the chest There is no agreement why the isthmus region is most exposed to ruptures

A discussion of the mechanism of traumatic rupture of the aorta must be based on an assessment of the forces acting on the aorta dur-

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THE MECHANISM OF TRAUMATIC RUPTURE OF THE AORTA

By

JON LUNDEVALL

Received 11 i 64

Traumatic rupture of the aorta may occur when the body, or the upper part of the body, is exposed to a sudden change of velocity by blunt force The ruptures usually occur in the isthmus region, more rarely in the ascending part of aorta, and seldom in other parts of the vessel The traumatic ruptures are nearly always transverse (For references, see *Lundevall* 1964)

The detailed mechanism which produces these ruptures have been discussed for many years, and opinions have in part been contradictory The aim of this study is to give a survey of the different views, and to contribute to the discussion by personal observations

Earlier Investigations

Rindfleisch (1893) held the opinion that traumatic rupture is produced by a sudden stretching of the vessel, and that the upper thoracic portion of aorta is most exposed to such stretching

Oppenheim (1918) ligated the branches of human aortas and filled the aortas with water at a pressure up to about 3000 mm Hg By this procedure, ruptures regularly occurred in the ascending part just above the semilunar valves In one instance there was a rupture at isthmus at 790 mm Hg pressure, but this particular aorta was calcified at this point In one perfectly healthy aorta two ruptures occurred, one just above the semilunar valves and one at isthmus, at a pressure of 2070 mm Hg Dissecting aneurysms frequently occurred during the experiment In two respects therefore, the ruptures caused by this procedure differed from those commonly observed during actual accidents Oppenheim admitted that a rise of the blood pressure to such high values hardly ever occurs in living persons, but he surmised that considerably lower internal pressures may cause rupture *in vivo* because the resistance of aorta against stress then is lower than after death The reason for this, he thought, was that tissue fluidization ceases at death, and that the body temperature is lowered

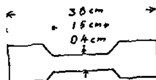


Fig 1
Dumbbell specimen

Observations on the Resistant Power of the Aortic Wall

Specimens of standardized measures (Dumbbell specimens, Fig 1) were punched from each of the 10 aortas already described

From each aorta, four specimens (Nos 1–4) were taken from the p asc, with their long axis along the blood stream, and symmetrically distributed around aorta's circumference The next four specimens (Nos 5–8) were from the isthmus region, and were also in symmetrical positions around the circumference, their long axis parallel to the blood stream The last two specimens (Nos 9–10) were from the posterior and the lateral parts of aorta's wall a little below isthmus, and were also taken from the lower thoracic portion of each of 2 aortas In each case, one specimen was longitudinal, the other transversal to the axis of the vessel, at the same level

Adventitia was removed from the specimens, and the thickness of the wall was measured with a micrometer in two specimens from each aorta, one from p asc, and one from the isthmus region

The specimens were stretched until they ruptured in an Instron stretching machine, kindly placed at disposal by the Norwegian Institute for Industrial Research The rate of stretching was the machine's fastest, 50 cm per minute The load at the moment of rupture was recorded The results are summarized in Table 2

TABLE 2

The Tensile Strength of the Wall of Aortas from 10 Bodies
Load (grams) of Dumbbell Specimens at Moment of Rupture

Body No	Spec no (p asc)				Spec no (isthmus)				Spec no (p desc)		Lower (p desc)	
	1	2	2	4	5	6	7	8	9	10	Long	Trv
I	530	490	730	400	490	420	360	190	450	320	410	895
II	375	735	825	455	505	215		315				
III	845	690	720	800			370	335	400	320		
IV	525	460	555	590	555	365	430	265	430	455		
V	565	440	540			485	235	200		240		
VI	690		545	490	355	250	365	310	315	365		
VII	675	440	490	565	535	430	390	320		650		
VIII		495	575	560		310	380	285				
IX	590	350	335	625		640	440	315	495	380	285	595
X	375	300	620	520	280	190	125	220	400	245		
Mean values			547 8				350 8				397 4	

ing velocity changes, and the ability of the aortic wall to resist these forces The following observations were made in order to obtain some data for this discussion

MATERIAL

Aortas from 10 bodies were studied The bodies were of 7 men and 3 women aged between 35 and 84 years Nine of the patients died of disease one (the youngest) of barbiturate poisoning All aortas presented various degrees of atherosclerosis but *none had calcifications of the aortic wall* The observations were made 1 2 days after death

RESULTS

Anatomical Observations

The course of the aortic arch deviated 20–25° from the sagittal plane In each case, the thoracic part of aorta was relatively mobile, but the isthmus region was slightly more fixated to its surroundings than the portions of aorta above and below The great arterial branches from the arch did not particularly restrain aorta's movements, nor did the intercostal arteries The arterial branches were easily stretched The recurrent nerve, running close to the concavity of the aortic arch at isthmus, was fairly resistant to traction In some instances the nerve was readily palpable through the aortic wall when aorta was pressed against the pulmonal root

The heart was relatively resistant against vertical movements, but was somewhat more mobile transversally, and could also to some extent be rotated along its long axis

The root of the left lung was relatively well fixed against any movements The anatomical measures of the aortas are given in Table 1

TABLE 1

Anatomical Measures of Aorta from 10 Bodies

Body			Arch diam cm	Aorta's radius cm		Thickness of wall cm	
No	Age (years)	Sex		p asc	isthmus	p asc	isthmus
I	35	♂	8	0.9	0.7	0.125	0.12
II	36	♂	6	1.0	0.8	0.155	0.165
III	39	♀	8	0.7	0.6	0.18	0.155
IV	47	♂	11.5	1.2	0.9	0.25	0.18
V	64	♂	10	1.4	1.1	0.215	0.2
VI	64	♀	8	1.3	1.0	0.14	0.14
VII	68	♂	10	0.9	0.6	0.18	0.13
VIII	79	♂	11	1.2	1.0	0.225	0.185
IX	80	♀	9	1.0	0.8	0.20	
X	84	♂	7.5	1.4	1.2	0.18	0.115
Mean values			8.9	1.1	0.87	0.185	0.167

Arch diameter is the horizontal distance between the outer (medial) wall of p asc and the outer (lateral) wall in the isthmus region at the level of the top of the concave wall of the arch

Aorta's radius at p asc and isthmus was calculated from the measured circumference

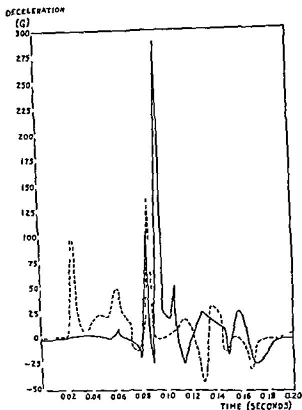


Fig 2

Deceleration/Time diagram (From Odelgard & Weman 1957)
--- Automobile ——— Shoulders

There are three kinds of mechanical forces which may act on the aortic wall during rapid velocity changes

A Traction and pressure on aorta by other thoracic organs, and torsion of the aorta

B Strain waves in the aortic wall

C Pressure variations in aorta s blood content

These forces will be discussed on the basis of the obeservations referred, and other author s obeservations

1 *The effect of traction and pressure on aorta by other thoracic organs and torsion of the aorta* From the anatomical observations it appears that during sudden changes of velocity in the sagittal plane (as in the example studied by *Odelgard & Weman* 1957) both the arch and the lower thoracic portion of the descending aorta are probably dislocated forwards or backwards in relation to the more fixed areas at isthmus and the base of the heart At these two points therefore,

During the tests, a few specimens were crushed at the fixation points, and were omitted as indicated in the table

During the slow stretching, the specimens were considerably elongated and reduced in thickness before they ruptured, an did not regain their original thickness when the stretching was discontinued The resistance of the specimens against the stretching was therefore mainly due to viscosity, not to the elasticity of the tissue

The resistant property of the wall varied considerably around the circumference at each level within each aorta However, the resistance was mainly less at isthmus than in the ascending and descending parts, and the transversal specimens resisted twice the load which caused rupture of the longitudinal specimens

DISCUSSION

1 Forces Acting on the Aortic Wall

A typical accident in which rupture may occur is the case of a motor car driver who is thrown against the steering wheel as the car collides frontally This situation has been studied experimentally by *Odelgard & Weman* (1957) In their experiment a dummy of anthropometric measures was rigged in the driver's seat of a car with a safety harness The car was dropped vertically and hit the ground at a speed of 60 km/h The movements of the car and the dummy were recorded, and derivations of the recordings gave, among others, the diagram shown in Fig 2

The dummy's shoulders were subjected to heavy vibrations lasting for about 0 12 seconds, during which decelerations up to 290 G occurred (G expresses the degree of change in velocity in multiples of magnitude of the acceleration of gravity) Other recordings showed that the hips of the dummy were also vibrated, but not synchronous with the shoulders The authors pointed out that, owing to the elastic response of the individual's body and the car's wheel, the magnitude of body vibrations on impact is difficult to predict It is reasonable, however, to assume that the absence of a damping harness will accentuate the peaks of deceleration, and also that these peaks will rise with the collision speed

The internal organs, including the aorta, will presumably also be exposed to vibrations during an impact The magnitude of these vibrations, however, is unknown at present because no experimental studies have been reported Some authors referred by *Zehnder* (1960), have made quantitative calculations of the degree of deceleration or acceleration necessary to produce rupture These calculations are based on the assumption that the change of velocity of the body, and of the aorta, is steady during an impact, but Odelgard & Weman s experiments have shown that this probably is not the case

authors already referred have stated that a sudden increase of the blood pressure is an important factor in the mechanism of traumatic rupture of the aorta no direct observations in this pressure have been reported The discussion of this factor must therefore be based on theoretical considerations

A local rise in the blood pressure may be caused in two ways Firstly by a local compression of aorta followed by displacement of the blood with an expansion of the adjacent uncompressed parts of aorta Secondly by the propagation of a pressure wave in the blood content causing a waterhammer effect

The first of these mechanisms is contingent upon the compression being applied for a time long enough to allow the blood to flow away from the compressed area and the rate of application of the pressure being so fast that only a limited area of the aortic wall at both sides of the compression is expanded *Moritz* (1932) found that pressures of 800 1000 mm Hg applied in the aorta of living rats caused rupture of the aorta or its branches Aorta did not rupture because the blood was led away too fast It is difficult to imagine that this mechanism of blood pressure increase can produce a single circular rupture as is usually seen in accident cases

The second mechanism is illustrated by the following example If a person during forward movement is suddenly stopped the blood in the aortic arch will continue forwards by its inertia and exert a pressure against the anterior wall of the ascending part of aorta and a corresponding traction on the posterior wall in the isthmus region The pressure is proportional to the weight of a horizontal column of blood lying close to the concave inner surface at the top of the arch and which has a base of 1 sq cm From the mean of measures of 10 aortas referred in Table 1 and the blood s specific weight it was calculated that this column weighs 9 4 g At a given degree of velocity change expressed in G s the pressure is therefore 9 4 G g sq cm

According to *Wehn* (1963) the resulting tension at different points of the aortic wall can be calculated in the following way

The aortic arch can be regarded as the half of a ring formed tube as shown in Fig 3

The ring s axis is the line AB lying in the pa...

...tance in the point E is found by tracing a diameter through this point and prolong it to the axis AB R is the distance CF and its plane of rotation is vertical to the paper s plane

Corresponding to the line FG the radius of curvature (R) shifts from $+\infty$ to $-\infty$ which means that R referring to points on the concave side of the tube s surface has a negative sign The point D therefore had a radius of curvature (R) equal to —CD

The formula for circular (meridional) stress (L_1) is (*Wehn* 1963)

local longitudinal stretchings of the aortic wall occur In cases in which the anterio-posterior diameter of the chest is reduced (as when the chest is pressed against the steering wheel), the heart will probably be dislocated to some extent to the left and also be rotated along its long axis A tangential torsion stress will then act on the aorta, preferentially on the part just above the semilunar valves, and tend to produce a spiral-formed rupture

During velocity changes along the vertical axis of the body (as when the individual falls on his feet or buttocks), the arch and the descending part of aorta will probably be dislocated downwards in relation to the isthmus region, producing a stretching of the upper lateral part of the wall at isthmus The arch will then be pressed against the left hilus, and the recurrent nerve may execute a cutting pressure from below

In traffic accidents, abdominal injuries often occur, causing an increased intra-abdominal pressure The diaphragm is often ruptured, and abdominal viscera are occasionally displaced into the left thoracic cavity The left lung's hilus is then pressed upwards, resulting in an increased bending, or even kinking of the aortic arch This mechanism will therefore tend to produce a transverse rupture in the isthmus region

The cervical vessels are stretched when the head is moved backwards, or when the aortic arch is pressed downwards Even if these vessels are easily stretched, they may exert a local traction on the aortic arch

In most accidents, the movements of the body are probably complex, the directions of velocity changes varying during fractions of a second Particularly in cases in which a pedestrian is hit by a car, he often tumbles head over heels, and it is virtually impossible to reconstruct which of the stresses discussed is the predominating in the mechanism causing aortic rupture It will also be apparent that the magnitude of the forces at present is incalculable

B Strain waves in the aortic wall Aldman (1962) has shown that when an elastic belt is stretched, a strain wave, which is a local elongation of the material, will start at one anchoring point of the belt The wave travels along the material and is reflected at the belt s ends If the belt is stretched faster than the material can be elongated the belt will rupture The lowest velocity at which rupture occurs by this effect is called the critical velocity, which can be estimated when the velocity of the strain waves are known

It may reasonably be assumed that similar strain waves occur in the elastic wall of aorta in accidents where a part of aorta is suddenly stretched As no experiments have been made to estimate this condition the importance of the phenomenon for the causation of rupture of the aorta is still unknown

C Pressure variations in aorta s blood content Although several

The formula for longitudinal stress in a straight tube is (*Windsor* 1959)

$$L_s = \frac{p\,r}{2}\ g/cm$$

and the corresponding tension (S_2) is

$$S_2 = \frac{p\,r}{2\,h}\ g/sq\ cm$$

Wehn (1963) has calculated that this tension has the same value in a curved tube (the hoop tension) as in a straight one, and the tension therefore, is independent of R

The numerical values for S_2 at different points were calculated from the data already referred, and were found to be

S_2 at isthmus 355 + 25 G g sq cm
S_2 at base 400 + 28 G g sq cm

In the outlined situation, the waterhammer effect will therefore cause a tension in the aortic wall which is greater in base than in the isthmus region, and the longitudinal tension (which *would produce a* transverse rupture) is about half as great as the circular tension

2 The Resistant Power of the Aortic Wall

Zehnder (1960), who measured the traction tolerance of specimens from aortas from 27 bodies found that the tolerance varied considerably among individuals, and also within each single aorta *The tolerance* was generally less against traction along the blood stream than against *transverse (circular)* traction Although the rate of onset of traction *was not stated*, the author concluded that the procentual elongation of the specimens *before they ruptured* expressed the elasticity of the aortic wall

In ideal experiments, the resistant *power of the* aortic wall should be tested during conditions similar to those occurring in actual accidents The tests should therefore be done on living tissue, and stress should be *applied* with a rate of onset and a duration as would occur in an accident This was *not feasible* during laboratory conditions, and for this reason a method *similar to* Zehnder's was employed

In the experiments *referred, only* the resistance due to viscosity of the tissue was measured *During actual* accidents, the stress on the aortic probably sets in so rapidly that there is no deformation of the wall before rupture *occurs* A resistance due to viscosity is then excluded, and the *experiments do only* coarsely reflect the resistant power of the aortic wall during such accidents

$$L_t = p\,r\left(1 - \frac{r}{2R}\right) \text{ g/cm}$$

where p is the pressure acting on the wall from the inside On the convex side L_t is less than p r, while on the concave side L_t is greater than p r The circular stress has a maximum on the concave side of the tube's wall and a minimum on the convex side

The tension on the wall (S_t) produced by the stress (L_t) is defined as the stress divided by the wall's thickness (h)

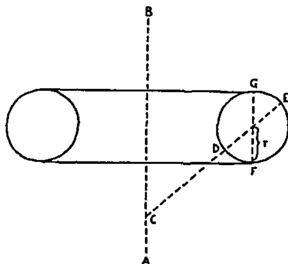


Fig 3
The aorta as a curved tube

For numerical evaluation of the circular tension caused by the water-hammer-effect in the mechanism outlined above, the mean values of R, r and h in Table 1 were used, although R and r probably are greater *in vivo* than post mortem, because of a possible postmortal contraction of aorta *The pressure p is the sum of the physiological blood pressure* (about 100 mm Hg or 136 g sq cm) and the additional pressure caused by the waterhammer-effect, 9 4 G g/sq cm

From the formula

$$S = \frac{p\,r}{h}\left(1 - \frac{r}{2R}\right)$$

the following values were found

$S_{t\ max}$	at concave side of isthmus	811 + 56 G g sq cm
$S_{t\ min}$	*at convex side of isthmus*	639 + 44 G g sq cm
$S_{t\ max}$	at concave side of p ase	956 + 66 G g/sq cm
$S_{t\ min}$	at convex side of p ase	709 + 49 G g/sq cm

region From the study of *Odelgard & Wieman* (1957) we know that in an autocar collision the driver's shoulders and hips undergo velocity changes of similar magnitude as those calculated above It is reasonable to assume that also the aorta may be exposed to velocity changes of such strength that a waterhammer effect is of importance in the mechanism causing rupture

The waterhammer effect alone would theoretically cause circular and longitudinal ruptures at about equal degrees of velocity change *Schnurbein* (1926) found that when increased internal pressure was applied in the straight portion of the descending aorta in rabbits the ruptures which occurred were longitudinal not transverse Why then are the ruptures occurring in aorta in actual accidents nearly always transverse? The reason probably is that the other forces already discussed which tend to cause transverse ruptures act together with the waterhammer mechanism

A study of the mechanism of traumatic rupture of the aorta is necessary for the choice of adequate countermeasures There is probably not much we can do to protect the pedestrian when he is hit by a fast moving car but the motorist driving a car which collides can be protected to a certain degree against this serious injury The aim of this protection must be to reduce the rate of deceleration and the development of adequate devices should be encouraged The ideal design of such devices depends on a precise knowledge of the mechanism which causes the damage

SUMMARY

The mechanism causing traumatic rupture of the aorta is discussed on the basis of earlier observations and personal studies The basis for the discussion is at present however insufficient and the conclusions must be drawn with caution

Of the different forces acting on the aortic wall only *the effect of a* pressure wave in aorta's blood content (a waterhammer mechanism) could be approximately calculated

Then tensile strength of the aortic wall of human bodies was tested under static conditions The results indicated that the isthmus *region* is less resistant to stress than the ascending and descending parts of aorta

On comparing the results it is concluded that rupture of the aorta is probably caused by the effect of forces *of different* kinds A waterhammer effect probably plays an important role but local pressure and traction and strain waves *in* the wall act simultaneously and explain why the ruptures are *nearly* always transverse and preferentially located in the isthmus region

Secondly, dead tissue probably has a higher power of resistance than living tissue (*Oppenheim* 1918 and personal observations) *Schnurbein* (1926) found that aortas of rabbits ruptured at about 1 3 atm internal pressure, whether the rabbits were freshly killed or already had a well developed rigor All his animals, however, were dead at the time of the experiments

These objections mainly concern the absolute values of resistance The measures probably give an approximately reliable picture of the relative resistant property of the different areas of each single aorta A calculation of the resistant power of the different parts of aorta, based on the mean values from Table 2, seems therefore justified

By derivation of the results it was found that the main resistant power of a cross section of the aortic wall against longitudinal traction is

at isthmus	5250 g/sq cm
at p asc	7400 g/sq cm

It was therefore concluded that the tissue of the aortic wall is somewhat less resistant to slow traction in the isthmus region than in p asc It was also presumed that the resistant power against transversal traction is the double of these figures in the respective areas, although this was measured in only two of the aortas

Comparison between damaging forces and the resistant power of the aortic wall From the anatomical observations it appears that traction and pressure on aorta by other thoracic organs, torsion of the aorta, and strain waves in the aortic wall during sudden stretching will mainly tend to produce transverse ruptures The observations indicate that the ruptures caused by these forces preferentially would occur in the isthmus region, but also in some case in the ascending part of aorta

In a certain situation, commonly occurring during accidents leading to rupture of a aorta, a waterhammer-effect caused by a pressure wave in the blood of the aortic arch must also be taken into consideration The amount of this effect rises with the degree of velocity change, and by derivation of the mean of the experimental results it was found that the waterhammer effect alone would produce rupture of the aortic wall at the following points, and at the following degrees of velocity change

Transverse rupture at isthmus	at 196 G
Longitudinal rupture at concave side of isthmus	173 G
Longitudinal rupture at convex side of isthmus	224 G
Transverse rupture in p asc	250 G
Longitudinal rupture at concave side of p asc	210 G
Longitudinal rupture at convex side of p asc	286 G

Although the figures are based on observations subject to criticism, it is reasonable to conclude that a somewhat higher degree of velocity change is necessary to produce rupture in p asc than in the isthmus

region From the study of *Odelgard & Wieman* (1957) we know that in an autocar collision the driver's shoulders and hips undergo velocity changes of similar magnitude as those calculated above It is reasonable to assume that also the aorta may be exposed to velocity changes of such strength that a waterhammer effect is of importance in the mechanism causing rupture

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Acta path et microbiol scandinav 62, 47 52, 1964

The Histological Department of the University of Uppsala Sweden

A FACTOR IN THE LIVER SCATTERING AGGREGATES OF LYMPHOID CELLS IN ARTIFICIALLY PREPARED SUSPENSIONS

By

K E FICHTELIUS

Received 27 ii 64

It was by chance observed that the number of cells in suspensions of thymus and lymph gland cells increased on the addition of liver extract Regardless of the type of liver factor concerned here, this observation sheds some light on the possible errors that may arise if the number of cells is calculated in artificial suspensions

THE NUMBER OF SUSPENDED LYMPHOID CELLS BEFORE AND AFTER THE PASSAGE OF GLASS BEADS COATED WITH ORGAN EXTRACTS

Experiment 1 A

In experiments involving the transfusion of P^{32}labelled thymus cells and lymph gland cells, *Fichtelius* (1958 a and b) demonstrated differences between the two types of lymphocytes Labelled thymus cells were traced to the liver and spleen of the receptor, while labelled lymph gland cells were traced to the liver and bone marrow In a later experiment with a different technique the lymph gland cells were also traced to the spleen, but not to the same extent as the thymus cells (*Diderholm & Fichtelius* 1959) The purpose of the following experiment was to demonstrate *in vitro* a similar difference between thymus and lymph gland cells

METHOD

Homogenates of spleen liver and bone marrow (equal parts of the organs and physiological saline or distilled water) were prepa[illegible]

[illegible] us suspension and lymph gland suspension were prepared as follows The thymus and lymph glands from a guinea pig were roughly freed of connective

The work has been supported by the *Swedish Medical Research Council*

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METHOD

Homogenates of spleen liver and bone marrow (equal parts of the organs and physiological saline or distilled water) were prepared from guinea pigs weighing approximately 200 g The homogenates were ... 00 g and the ... were sul m... at lea... for The g... ing to the extr... burettes with height of 30 c...

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tissue washed in Earl's solution their surfaces were carefully freed of remaining connective tissue and they were then washed again in Earl's solution The organs were then cut up finely and placed into a glass vessel 6 ml of Earl's solution were added and the pieces of tissue were stirred vigorously with a forceps or scissor blades and pressed against the sides of the glass vessel The tissue mixture was then sieved through ordinary muslin

2 ml of the thymus suspension and lymph gland suspension respectively were poured into each of the burettes with spleen liver and bone marrow beads and were allowed to pass through 5 5 cc of these, after which the tap was closed 1 minute after the start the tap was opened and the suspension was allowed to pass through a further 0 5 cc of beads after which the tap was closed again Every succeeding minute the suspension was passed through 0 5 cc of 'new' beads in this way At 9 minutes after the start the suspension was let down to the 10 cc mark and at 10 minutes it was released through the tap and the first three drops taken for examination The number of cells per cmm in the suspension before and after its passage through the beads was then counted (dilution 1 20 or 1 100 2 × 32 B squares counted in the Burker chamber) The number per cmm after the passage was given as a percentage of the number per cmm beofre Three thymus suspensions and three lymph gland suspensions were passed through glass beads coated with saline extracts, and four suspensions of each kind through glass beads coated with water extracts

RESULTS

As can be seen in Table 1, there were fewer cells per cmm after passage through spleen and bone marrow beads On the other hand there were more cells per cmm after passage through liver beads No significant difference were observed between the thymus and lymph gland cells

TABLE 1

No of Cells per cmm after the Passage through Glass Beads Coated with Extract as a Percentage of the Number per cmm before Passage

	Spleen beads	Liver beads	Bone marrow beads
Saline extract			
Thymus suspension	90 2	96 3	72 0
	103 9	182 4	68 6
	76 4	156 9	44 4
Mean	90 2	145 2	61 7
Lymph gland suspension	88 2	163 2	111 8
	93 2	106 8	50 0
	101 7	230 5	74 6
Mean	94 4	166 8	78 8
Water extract			
Thymus suspension	48 9	118 9	91 1
	56 1	129 0	61 3
	61 9	228 6	66 6
	88 2	202 0	80 4
Mean	63 8	169 6	74 9
Lymph gland suspension	44 9	146 9	67 3
	45 5	236 4	36 4
	51 3	200 0	74 4
	58 3	291 7	83 3
Mean	50 0	218 8	65 4

The study confirms earlier observations that these ruptures occur independent of age and pathologic changes of the aorta. Middle aged men form the largest group probably because they most often expose themselves to serious traffic accidents. In contrast to earlier observations the material contains more motorists than pedestrians. This is probably because most accidents happened on country roads where as civilization proceeds pedestrians are a vanishing race.

Traumatic rupture of the aorta is usually accompanied by fatal intrathoracic haemorrhage. The survival time is only in a few instances long enough to permit surgical treatment. In a number of cases this injury is the single serious lesion in cases of traffic accidents. There are therefore ample reasons to analyse, as far as possible the mechanical forces acting in these instances in order to find adequate counter measures. Such analysis is the subject of another study (*Lundevall* 1964).

SUMMARY

Twenty one cases of fatal traumatic ruptures of aorta are reported. All cases except one were traffic accidents. Middle aged men were the largest group of the injured. The ruptures were predominantly located at the isthmus of aorta. In 16 cases only one rupture occurred, in 5 cases there were 2 or more ruptures. Severe intrathoracic haemorrhage was present in all cases and probably represented the single cause of death in 5 cases.

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Institute of Forensic Medicine, Oslo University
(Head: Professor Georg Waaler)

THE MECHANISM OF TRAUMATIC RUPTURE OF THE AORTA

By

JON LUNDEVALL

Received 11.i.64

Traumatic rupture of the aorta may occur when the body, or the upper part of the body, is exposed to a sudden change of velocity by blunt force The ruptures usually occur in the isthmus region, more rarely in the ascending part of aorta, and seldom in other parts of the vessel The traumatic ruptures are nearly always transverse (For references, see *Lundevall* 1964)

The detailed mechanism which produces these ruptures have been discussed for many years, and opinions have in part been contradictory The aim of this study is to give a survey of the different views, and to contribute to the discussion by personal observations

Earlier Investigations

Rindfleisch (1893) held the opinion that traumatic rupture is produced by a sudden stretching of the vessel, and that the upper thoracic portion of aorta is most exposed to such stretching

Oppenheim (1918) ligated the branches of human aortas and filled the aortas with water at a pressure up to about 3000 mm Hg By this procedure, ruptures regularly occurred in the ascending part just above the semilunar valves In one instance there was a rupture at isthmus at 790 mm Hg pressure, but this particular aorta was calcified at this point In one perfectly healthy aorta two ruptures occurred, one just above the semilunar valves and one at isthmus, at a pressure of 2070 mm Hg Dissecting aneurysms frequently occurred during the experiment In two respects therefore, the ruptures caused by this procedure differed from those commonly observed during actual accidents Oppenheim admitted that a rise of the blood pressure to such high values hardly ever occurs in living persons, but he surmised that considerably lower internal pressures may cause rupture *in vivo* because the resistance of aorta against stress then is lower than after death The reason for this, he thought, was that tissue fluidization ceases at death, and that the body temperature is lowered

Oppenheim did not state at which rate the pressures were applied, but it is inferred from his remarks that this was rather slow.

Klotz & Simpson (1932) found in similar experiments that aorta from young persons resisted an internal pressure of 1000 mm Hg without rupturing, but they nevertheless assumed that it is the sudden rise of the blood pressure which ruptures the aorta during accidents. Even *Kleinsasser* (1943), *Strassmann* (1947) and *Tannenbaum & Ferguson* (1948) were of the opinion that a rise in blood pressure is an important factor for the occurrence of these ruptures.

No experiments have been reported in which the blood pressure in the thoracic portion of aorta has been measured during impacts to the body.

A number of authors have stressed the importance of aorta's topography in order to explain the mechanism of rupture. *Letterer* (1924) thought that in cases where the individual fell on his feet or buttocks from a height, the rupture was caused by the heart's downward traction on the aorta.

Shennan (1929) held the opinion that the relative fixation of the aorta to its surroundings at isthmus had a "hinge" effect, causing rupture at this point by sudden movements of the chest, and *Kleinsasser* (1943) sustained this opinion.

Hass (1944) more generally stated that any rapid relative displacement of two adjacent parts of the body causes a stress at the junction, and that this is the basis for the mechanism of aortic rupture. *Tannenbaum & Ferguson* (1948) agreed to some extent, but said that some cases of traumatic rupture of the aorta could not be explained by Hass' theory, as for instance thoses cases in which rupture is caused by flying pieces of wood or stone, hitting the chest.

Marshall (1938) pointed out that when the body is decelerated during forward motion, the hilus of the left lung will exert a traction on the parietal pleura covering the aorta. By this mechanism aorta is probably kinked in such a way that rupture occurs.

Cammack et al (1959) found that anterio posterior compression of the chest will cause a dislocation of the heart to the left, and a torsion of the aorta with a maximal stress on its wall near the base of the heart.

Zehnder (1960) deduced from theoretical considerations that the main factor for different traumata is a hyperflexion of the aortic arch, with a predominant stress on the isthmus region.

To summarize, earlier authors agree that traumatic rupture occurs in connection with a considerable and sudden change of velocity of the body. The direction of the velocity change may vary from one case to another and is often but not always accompanied by a contusion of the chest. There is no agreement why the isthmus region is most exposed to ruptures.

A discussion of the mechanism of traumatic rupture of the aorta must be based on an assessment of the forces acting on the aorta dur-

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Earlier Investigations

Rindfleisch (1893) held the opinion that traumatic rupture is produced by a sudden stretching of the vessel, and that the upper thoracic portion of aorta is most exposed to such stretching

Oppenheim (1918) ligated the branches of human aortas and filled the aortas with water at a pressure up to about 3000 mm Hg By this procedure, ruptures regularly occurred in the ascending part just above the semilunar valves In one instance there was a rupture at isthmus at 790 mm Hg pressure, but this particular aorta was calcified at this point In one perfectly healthy aorta two ruptures occurred, one just above the semilunar valves and one at isthmus, at a pressure of 2070 mm Hg Dissecting aneurysms frequently occurred during the experiment In two respects therefore, the ruptures caused by this procedure differed from those commonly observed during actual accidents Oppenheim admitted that a rise of the blood pressure to such high values hardly ever occurs in living persons, but he surmised that considerably lower internal pressures may cause rupture *in vivo* because the resistance of aorta against stress then is lower than after death The reason for this, he thought, was that tissue fluidization ceases at death, and that the body temperature is lowered

Oppenheim did not state at which rate the pressures were applied, but it is inferred from his remarks that this was rather slow

Klotz & Simpson (1932) found in similar experiments that aorta from young persons resisted an internal pressure of 1000 mm Hg without rupturing, but they nevertheless assumed that it is the sudden rise of the blood pressure which ruptures the aorta during accidents Even *Kleinsasser* (1943), *Strassmann* (1947) and *Tannenbaum & Ferguson* (1948) were of the opinion that a rise in blood pressure is an important factor for the occurrence of these ruptures

No experiments have been reported in which the blood pressure in the thoracic portion of aorta has been measured during impacts to the body

A number of authors have stressed the importance of aorta's topography in order to explain the mechanism of rupture *Letterer* (1924) thought that in cases where the individual fell on his feet or buttocks from a height, the rupture was caused by the heart's downward traction on the aorta

Shennan (1929) held the opinion that the relative fixation of the aorta to its surroundings at isthmus had a "hinge" effect, causing rupture at this point by sudden movements of the chest, and *Kleinsasser* (1943) sustained this opinion

Hass (1944) more generally stated that any rapid relative displacement of two adjacent parts of the body causes a stress at the junction, and that this is the basis for the mechanism of aortic rupture *Tannenbaum & Ferguson* (1948) agreed to some extent, but said that some cases of traumatic rupture of the aorta could not be explained by Hass' theory, as for instance thoses cases in which rupture is caused by flying pieces of wood, or stone, hitting the chest

Marshall (1938) pointed out that when the body is decelerated during forward motion, the hilus of the left lung will exert a traction on the parietal pleura covering the aorta By this mechanism aorta is probably kinked in such a way that rupture occurs

Cammack et al (1959) found that anterio posterior compression of the chest will cause a dislocation of the heart to the left, and a torsion of the aorta with a maximal stress on its wall near the base of the heart

Zehnder (1960) deduced from theoretical considerations that the main factor for different traumata is a hyperflexion of the aortic arch, with a predominant stress on the isthmus region

To summarize, earlier authors agree that traumatic rupture occurs in connection with a considerable and sudden change of velocity of the body The direction of the velocity change may vary from one case to another, and is often but not always accompanied by a contusion of the chest There is no agreement why the isthmus region is most exposed to ruptures

A discussion of the mechanism of traumatic rupture of the aorta must be based on an assessment of the forces acting on the aorta dur-

ing velocity changes, and the ability of the aortic wall to resist these forces The following observations were made in order to obtain some data for this discussion

MATERIAL

Aortas from 10 bodies were studied The bodies were of 7 men and 3 women aged between 35 and 84 years Nine of the patients died of disease, one (the youngest) of barbiturate poisoning All aortas presented various degrees of atherosclerosis but none had calcifications of the aortic wall The observations were made 1 2 days after death

RESULTS

Anatomical Observations

The course of the aortic arch deviated 20–25° from the sagittal plane In each case, the thoracic part of aorta was relatively mobile, but the isthmus region was slightly more fixated to its surroundings than the portions of aorta above and below The great arterial branches from the arch did not particularly restrain aorta's movements, nor did the intercostal arteries The arterial branches were easily stretched The recurrent nerve, running close to the concavity of the aortic arch at isthmus, was fairly resistant to traction In some instances the nerve was readily palpable through the aortic wall when aorta was pressed against the pulmonal root

The heart was relatively resistant against vertical movements, but was somewhat more mobile transversally, and could also to some extent be rotated along its long axis

The root of the left lung was relatively well fixed against any movements The anatomical measures of the aortas are given in Table 1

TABLE 1
Anatomical Measures of Aorta from 10 Bodies

Body			Arch diam cm	Aorta's radius cm		Thickness of wall cm	
No	Age (years)	Sex		p asc	isthmus	p asc	isthmus
I	35	♂	8	0 9	0 7	0 125	0 12
II	36	♂	6	1 0	0 8	0 155	0 165
III	39	♀	8	0 7	0 6	0 18	0 155
IV	47	♂	11 5	1 2	0 9	0 25	0 18
V	64	♂	10	1 4	1 1	0 215	0 2
VI	64	♀	8	1 3	1 0	0 14	0 14
VII	68	♂	10	0 9	0 6	0 18	0 13
VIII	79	♂	11	1 2	1 0	0 225	0 185
IX	80	♀	9	1 0	0 8	0 20	
X	84	♂	7 5	1 4	1 2	0 18	0 115
Mean values			8 9	1 1	0 87	0 185	0 167

Arch diameter is the horizontal distance between the outer (medial) wall of p asc and the outer (lateral) wall in the isthmus region at the level of the top of the concave wall of the arch

Aorta's radius at p asc and isthmus was calculated from the measured circumference



Fig 1
Dumbbell specimen

Observations on the Resistant Power of the Aortic Wall

Specimens of standardized measures (Dumbbell specimens, Fig 1) were punched from each of the *10 aortas already described*

From each aorta, four specimens (Nos 1–4) were taken from the p asc, with their long axis along the blood stream, and symmetrically distributed around aorta's circumference The next four specimens (Nos 5–8) were from the isthmus region, and were also in symmetrical positions around the circumference, their *long axis parallel to the blood* stream The last two specimens (Nos 9–10) were from the posterior and the lateral parts of aorta's wall a little below isthmus, and were also taken from the lower thoratic portion of each of 2 aortas In each case, one specimen was longitudinal, the other transversal to the axis of the vessel, at the same level

Adventitia was removed from the specimens, and the thickness of the wall was measured with a micrometer in two *specimens from each* aorta, one from p asc, and one from the isthmus region

The specimens were stretched until they ruptured in an Instron stretching machine, kindly placed at disposal by the Norwegian Institute for Industrial Research The rate of stretching was the machine's fastest, 50 cm *per minute The load at the moment of rupture* was recorded The results are summarized in Table 2

TABLE 2

The Tensile Strength of the Wall of Aortas from 10 Bodies
Load (grams) of Dumbbell Specimens at Moment of Rupture

Body No	Spec no (p asc)				Spec no (isthmus)				Spec no (p desc)		Lower (p desc)	
	1	2	3	4	5	6		8	9	10	Long	Trv
I	530	490	730	400	490	420	360	190	450	320	410	895
II	375	735	825	455	505	215		345				
III	845	690	720	800			370	335	400	320		
IV	525	460	555	590	555	365	430	265	430	455		
V	565	440	540			485	235	200		240		
VI	690		545	490	355	250	365	310	315	365		
VII	675	440	490	565	535	430	390	320		650		
VIII		495	575	560		310	380	285				
IX	590	350	335	625		640	440	315	495	380	285	595
X	375	300	620	520	280	190	125	220	400	245		
Mean values			547 8				350 6			397 4		

ing velocity changes, and the ability of the aortic wall to resist these forces. The following observations were made in order to obtain some data for this discussion.

MATERIAL

Aortas from 10 bodies were studied. The bodies were of 7 men and 3 women aged between 35 and 84 years. Nine of the patients died of disease, one (the youngest) of barbiturate poisoning. All aortas presented various degrees of atherosclerosis, but none had calcifications of the aortic wall. The observations were made 1–2 days after death.

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TABLE 1
Anatomical Measures of Aorta from 10 Bodies

Body			Arch diam. cm	Aorta's radius cm		Thickness of wall cm	
No.	Age (years)	Sex		p. asc.	isthmus	p. asc.	isthmus
I	35	♂	8	0.9	0.7	0.125	0.12
II	36	♂	6	1.0	0.8	0.155	0.165
III	39	♀	8	0.7	0.6	0.18	0.155
IV	47	♂	11.5	1.2	0.9	0.25	0.18
V	64	♂	10	1.4	1.1	0.215	0.2
VI	64	♀	8	1.3	1.0	0.14	0.14
VII	68	♂	10	0.9	0.6	0.18	0.13
VIII	79	♂	11	1.2	1.0	0.225	0.185
IX	80	♀	9	1.0	0.8	0.20	
X	84	♂	7.5	1.4	1.2	0.18	0.115
Mean values			8.9	1.1	0.87	0.185	0.167

Arch diameter is the horizontal distance between the outer (medial) wall of p. asc. and the outer (lateral) wall in the isthmus region, at the level of the top of the concave wall of the arch.

Aorta's radius at p. asc. and isthmus was calculated from the measured circumference.

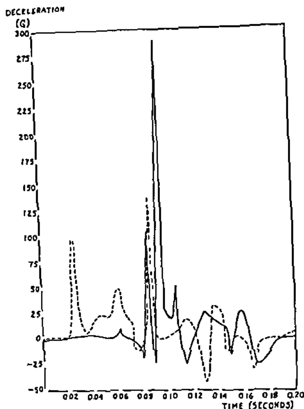


Fig 2

Deceleration/Time diagram (From Odelgard & Weman 1957)
----- Automobile ——— Shoulders

There are three kinds of mechanical forces which may act on the aortic wall during rapid velocity changes:

A Traction and pressure on aorta by other thoracic organs, and torsion of the aorta

B Strain waves in the aortic wall

C Pressure variations in aorta's blood content

These forces will be discussed on the basis of the obeservations referred, and other author's obeservations

A The effect of traction and pressure on aorta by other thoracic organs and torsion of the aorta From the anatomical observations it appears that during sudden changes of velocity in the sagittal plane (as in the example studied by *Odelgard & Weman* 1957) both the arch and the lower thoracic portion of the descending aorta are probably dislocated forwards or backwards in relation to the more fixed areas at isthmus and the base of the heart At these two points therefore,

During the tests, a few specimens were crushed at the fixation points, and were omitted as indicated in the table

During the slow stretching, the specimens were considerably elongated and reduced in thickness before they ruptured, an did not regain their original thickness when the stretching was discontinued The resistance of the specimens against the stretching was therefore mainly due to viscosity, not to the elasticity of the tissue

The resistant property of the wall varied considerably around the circumference at each level within each aorta However, the resistance was mainly less at isthmus than in the ascending and descending parts, and the transversal specimens resisted twice the load which caused rupture of the longitudinal specimens

DISCUSSION

1 *Forces Acting on the Aortic Wall*

A typical accident in which rupture may occur is the case of a motor car driver who is thrown against the steering wheel as the car collides frontally This situation has been studied experimentally by *Odelgard & Weman* (1957) In their experiment a dummy of anthropometric measures was rigged in the driver's seat of a car with a safety harness The car was dropped vertically and hit the ground at a speed of 60 km/h The movements of the car and the dummy were recorded, and derivations of the recordings gave, among others, the diagram shown in Fig 2

The dummy's shoulders were subjected to heavy vibrations lasting for about 0 12 seconds, during which decelerations up to 290 G occurred (G expresses the degree of change in velocity in multiples of magnitude of the acceleration of gravity) Other recordings showed that the hips of the dummy were also vibrated but not synchronous with the shoulders The authors pointed out that, owing to the elastic response of the individual's body and the car s wheel, the magnitude of body vibrations on impact is difficult to predict It is reasonable, however, to assume that the absence of a damping harness will accentuate the peaks of deceleration, and also that these peaks will rise with the collision speed

The internal organs including the aorta, will presumably also be exposed to vibrations during an impact The magnitude of these vibrations, however, is unknown at present because no experimental studies have been reported Some authors referred by *Zehnder* (1960) have made quantitative calculations of the degree of deceleration or acceleration necessary to produce rupture These calculations are based on the assumption that the change of velocity of the body, and of the aorta is steady during an impact, but Odelgard & Weman s experiments have shown that this probably is not the case

authors already referred have stated that a sudden increase of the blood pressure is an important factor in the mechanism of traumatic rupture of the aorta no direct observations in this pressure have been reported The discussion of this factor must therefore be based on theoretical considerations

A local rise in the blood pressure may be caused in two ways Firstly by a local compression of aorta followed by displacement of the blood with an expansion of the adjacent uncompressed parts of aorta Secondly by the propagation of a pressure wave in the blood content causing a waterhammer-effect

The first of these mechanisms is contingent upon the compression being applied for a time long enough to allow the blood to flow away from the compressed area and the rate of application of the pressure being so fast that only a limited area of the aortic wall at both sides of the compression is expanded *Moritz* (1932) found that pressures of 800 1000 mm Hg applied in the aorta of living rats caused rupture of the aorta or its branches Aorta did not rupture because the blood was led away too fast It is difficult to imagine that this mechanism of blood pressure increase can produce a single circular rupture as is usually seen in accident cases

The second mechanism is illustrated by the following example If a person during forward movement is suddenly stopped the blood in the aortic arch will continue forwards by its inertia and exert a pressure against the anterior wall of the ascending part of aorta and a corresponding traction on the posterior wall in the isthmus region The pressure is proportional to the weight of a horizontal column of blood lying close to the concave inner surface at the top of the arch and which has a base of 1 sq cm From the mean of measures of 10 aortas referred in Table 1 and the blood s specific weight it was calculated that this column weighs 9 4 g At a given degree of velocity change expressed in G s the pressure is therefore 9 4 G g sq cm

According to *Wehn* (1963) the resulting tension at different points of the aortic wall can be calculated in the following way

The aortic arch can be regarded as the half of a ring formed tube as shown in Fig 3

The ring s axis is the line AB lying in the paper s plane Each [illegible]
on th[illegible]
of re[illegible]
curv[illegible] distance in the point E is found by *tracing a diameter* through this point and prolong it to the axis AB R is the distance CF and its plane of rotation is vertical to the paper s plane

Corresponding to the line FG the radius of curvature (R) shifts from + ∞ to − ∞ which means that R referring to points on the concave side of the tube s surface has a negative sign The point D therefore has a radius of curvature (R) equal to —CD

The formula for circular (meridional) stress (T_1) is (*Wehn* 1963)

local longitudinal stretchings of the aortic wall occur In cases in which the anterio posterior diameter of the chest is reduced (as when the chest is pressed against the steering wheel), the heart will probably be dislocated to some extent to the left and also be rotated along its long axis A tangential torsion stress will then act on the aorta, preferentially on the part just above the semilunar valves, and tend to produce a spiral formed rupture

During velocity changes along the vertical axis of the body (as when the individual falls on his feet or buttocks), the arch and the descending part of aorta will probably be dislocated downwards in relation to the isthmus region, producing a stretching of the upper lateral part of the wall at isthmus The arch will then be pressed against the left hilus, and the recurrent nerve may execute a cutting pressure from below

In traffic accidents, abdominal injuries often occur, causing an increased intra abdominal pressure The diaphragm is often ruptured, and abdominal viscera are occasionally displaced into the left thoracic cavity The left lung's hilus is then pressed upwards, resulting in an increased bending, or even kinking of the aortic arch This mechanism will therefore tend to produce a transverse rupture in the isthmus region

The cervical vessels are stretched when the head is moved backwards, or when the aortic arch is pressed downwards Even if these vessels are easily stretched, they may exert a local traction on the aortic arch

In most accidents, the movements of the body are probably complex, the directions of velocity changes varying during fractions of a second Particularly in cases in which a pedestrian is hit by a car, he often tumbles *head over heels, and it is virtually impossible to reconstruct* which of the stresses discussed is the predominating in the mechanism causing aortic rupture It will also be apparent that the magnitude of the forces at present is incalculable

B Strain waves in the aortic wall Aldman (1962) has shown that when an elastic belt is stretched, a strain wave, which is a local elongation of the material, will start at one anchoring point of the belt The wave travels along the material and is reflected at the belt's ends If the belt is stretched faster than the material can be elongated, the belt will rupture The lowest velocity at which rupture occurs by this effect is called the critical velocity, which can be estimated when the velocity of the strain waves are known

It may reasonably be assumed that similar strain waves occur in the elastic wall of aorta in accidents where a part of aorta is suddenly stretched As no experiments have been made to estimate this condition, the importance of the phenomenon for the causation of rupture of the aorta is still unknown

C Pressure variations in aorta s blood content Although several

The formula for longitudinal stress in a straight tube is (Windsor 1959)

$$L_{\cdot} = \frac{p\,r}{2}\ \text{g/cm}$$

and the corresponding tension (S) is

$$S_{\cdot} = \frac{p\,r}{2\,h}\ \text{g sq cm}$$

Wehn (1963) has calculated that this tension has the same value in a curved tube (the hoop tension) as in a straight one and the tension therefore is independent of R

The numerical values for S at different points were calculated from the data already referred and were found to be

S at isthmus 355 + 25 G g sq cm
S at p asc 400 + 28 G g sq cm

In the outlined situation the waterhammer effect will therefore cause a tension in the aortic wall which is greater in p asc than in the isthmus region and the longitudinal tension (which would produce a transverse rupture) is about half as great as the circular tension

' *The Resistant Power of the Aortic Wall*

Zehnder (1960) who measured the traction tolerance of specimens from aortas from 97 bodies found that the tolerance varied considerably among individuals and also within each single aorta The tolerance was generally less against traction along the blood stream than against transverse (circular) traction Although the rate of onset of traction was not stated the author concluded that the procentual elongation of the specimens before they ruptured expressed the elasticity of the aortic wall

In ideal experiments the resistant power of the aortic wall should be tested during conditions similar to those occurring in actual accidents The tests should therefore be done on living tissue and stress should be applied with a rate of onset and a duration as would occur in an accident This was not feasible during laboratory conditions and for this reason a method similar to Zehnder s was employed

In the experiments referred only the resistance due to viscosity of the tissue was measured During actual accidents the stress on the aortic probably sets in so rapidly that there is no deformation of the wall before rupture occurs A resistance due to viscosity is then excluded and the experiments do only coarsely reflect the resistant power of the aortic wall during such accidents

$$L_1 = p\,r\left(1 - \frac{r}{2\,R}\right) \text{ g/cm}$$

where p is the pressure acting on the wall from the inside. On the convex side L_1 is less than p r, while on the concave side L_1 is greater than p r. The circular stress has a maximum on the concave side of the tube's wall and a minimum on the convex side.

The tension on the wall (S_1) produced by the stress (L_1) is defined as the stress divided by the wall's thickness (h).

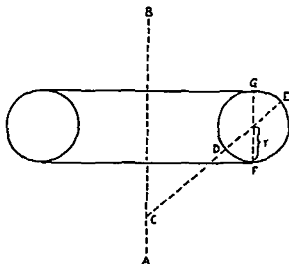


Fig 3
The aorta as a curved tube

For numerical evaluation of the circular tension caused by the waterhammer-effect in the mechanism outlined above, the mean values of R, r and h in Table 1 were used, although R and r probably are greater *in vivo* than post mortem, because of a possible postmortal contraction of aorta The pressure p is the sum of the physiological blood pressure (about 100 mm Hg or 136 g/sq cm) and the additional pressure caused by the waterhammer-effect, 9 4 G g/sq cm.

From the formula

$$S = \frac{p\,r}{h}\left(1 - \frac{r}{2\,R}\right)$$

the following values were found

$S_{1\,max}$	at concave side of isthmus ·	811 + 56 G g/sq cm
$S_{1\,min}$	at convex side of isthmus	639 + 44 G g/sq cm
$S_{1\,max}$	at concave side of p asc	956 + 66 G g/sq cm
$S_{1\,min}$	at convex side of p asc	709 + 49 G g sq cm

region From the study of *Odelgard & Weman* (1957) we know that, in an autocar collision, the driver's shoulders and hips undergo velocity changes of similar magnitude as those calculated above It is reasonable to assume that also the aorta may be exposed to velocity changes of such strength that a waterhammer effect is of importance in the mechanism causing rupture

The waterhammer-effect alone would theoretically cause circular and longitudinal ruptures at about equal degrees of velocity change *Schnurbein* (1926) found that when increased internal pressure was applied in the straight portion of the descending aorta in rabbits, the ruptures which occurred were longitudinal, not transverse Why then, are the ruptures occurring in aorta in actual accidents nearly always transverse? The reason probably is that the other forces already discussed, which tend to cause transverse ruptures, act together with the waterhammer mechanism

A study of the mechanism of traumatic rupture of the aorta is necessary for the choice of adequate countermeasures There is probably not much we can do to protect the pedestrian when he is hit by a fast-moving car, but the motorist driving a car which collides can be protected to a certain degree against this serious injury The aim of this protection must be to reduce the rate of deceleration, and the development of adequate devices should be encouraged The ideal design of such devices depends on a precise knowledge of the mechanism which causes the damage

SUMMARY

The mechanism causing traumatic rupture of the aorta is discussed on the basis of earlier observations and personal studies The basis for the discussion is at present, however, insufficient, and the conclusions must be drawn with caution

Of the different forces acting on the aortic wall, only the effect of a pressure wave in aorta's blood content (a waterhammer-mechanism) could be approximately calculated

Then tensile strength of the aortic wall of human bodies was tested under static conditions The results indicated that the isthmus region is less resistant to stress than the ascending and descending parts of aorta

On comparing the results it is concluded that rupture of the aorta is probably caused by the effect of forces of different kinds A waterhammer effect probably plays an important rôle, but local pressure and traction and strain waves in the wall act simultaneously and explain why the ruptures are nearly always transverse, and preferentially located in the isthmus region

Secondly, dead tissue probably has a higher power of resistance than living tissue (*Oppenheim* 1918 and personal observations) *Schnurbein* (1926) found that aortas of rabbits ruptured at about 1 3 atm internal pressure, whether the rabbits were freshly killed or already had a well developed rigor All his animals, however, were dead at the time of the experiments

These objections mainly concern the absolute values of resistance The measures probably give an approximately reliable picture of the relative resistant property of the different areas of each single aorta A calculation of the resistant power of the different parts of aorta, based on the mean values from Table 2, seems therefore justified

By derivation of the results it was found that the main resistant power of a cross section of the aortic wall against longitudinal traction is

at isthmus	5250 g/sq cm
at p asc	7400 g/sq cm

It was therefore concluded that the tissue of the aortic wall is somewhat less resistant to slow traction in the isthmus region than in p asc It was also presumed that the resistant power against transversal traction is the double of these figures in the respective areas, although this was measured in only two of the aortas

Comparison between damaging forces and the resistant power of the aortic wall From the anatomical observations it appears that traction and pressure on aorta by other thoracic organs, torsion of the aorta and strain waves in the aortic wall during sudden stretching will mainly tend to produce transverse ruptures The observations indicate that the ruptures caused by these forces preferentially would occur in the isthmus region, but also in some case in the ascending part of aorta

In a certain situation, commonly occurring during accidents leading to rupture of a aorta, a waterhammer-effect caused by a pressure wave in the blood of the aortic arch must also be taken into consideration The amount of this effect rises with the degree of velocity change, and by derivation of the mean of the experimental results it was found that the waterhammer-effect alone would produce rupture of the aortic wall at the following points, and at the following degrees of velocity change

Transverse rupture at isthmus	at 196 G
Longitudinal rupture at concave side of isthmus	173 G
Longitudinal rupture at convex side of isthmus	224 G
Transverse rupture in p asc	250 G
Longitudinal rupture at concave side of p asc	210 G
Longitudinal rupture at convex side of p asc	286 G

Although the figures are based on observations subject to criticism, it is reasonable to conclude that a somewhat higher degree of velocity change is necessary to produce rupture in p asc than in the isthmus

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The Histological Department of the University of Uppsala, Sweden

A FACTOR IN THE LIVER SCATTERING AGGREGATES OF LYMPHOID CELLS IN ARTIFICIALLY PREPARED SUSPENSIONS

By

K E Fichtelius

Received 27 II 64

It was by chance observed that the number of cells in suspensions of thymus and lymph gland cells increased on the addition of liver extract Regardless of the type of liver factor concerned here, this observation sheds some light on the possible errors that may arise if the number of cells is calculated in artificial suspensions

THE NUMBER OF SUSPENDED LYMPHOID CELLS BEFORE AND AFTER THE PASSAGE OF GLASS BEADS COATED WITH ORGAN EXTRACTS

Experiment 1 A

In experiments involving the transfusion of P³²labelled thymus cells and lymph gland cells, *Fichtelius* (1958 a and b) demonstrated differences between the two types of lymphocytes Labelled thymus cells were traced to the liver and spleen of the receptor, while labelled lymph gland cells were traced to the liver and bone marrow In a later experiment with a different technique the lymph gland cells were also traced to the spleen, but not to the same extent as the thymus cells (*Diderholm & Fichtelius* 1959) The purpose of the following experiment was to demonstrate *in vitro* a similar difference between thymus and lymph gland cells

METHOD

Homogenates of spleen liver and bone marrow (equal parts of the organs and physiological saline or distilled water) were prepared from guinea pigs weighing approximately 200 g The homogenates were centrif [illegible]

[illegible] thymus suspension and lymph gland suspension were prepared as follows The thymus and lymph glands from a guinea pig were roughly freed of connective

The work has been supported by the *Swedish Medical Research Council*

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Experiment 1 B

In experiment 1 A the change in the number of cells per cmm as a result of passage through glass beads coated with extract was investigated, in experiment 1 B the technique was modified so that the total number entering and leaving the column could be counted

METHOD

The preparation of suspensions and beads coated with extract was carried out as in experiment 1 A The extract was prepared with distilled water When at 10 minutes after the start the suspensions were allowed to run out through the tap 2 ml of Earl s solution were added The tap was left open for the following 5 minutes The fluid collected was measured carefully The total number of cells that were introduced among the beads and the number collected after passage through them were counted with the aid of the Burker chamber The number that passed through was given as a percentage of those allowed to come in 9 separations were performed with thymus suspensions and 9 with lymph gland suspensions

RESULTS

As may be seen in Table 2, between 50 and 70 per cent of the cells passed through the spleen and bone marrow beads No difference was observed between the thymus and lymph gland cells On the other hand there were more cells in the fluid collected after the passage through the liver beads than in the 2 ml of suspension that was poured in The thymus and lymph gland cells behaved similarly in this respect

TABLE 2

The Passage of Cells through Glass Beads coated with Extracts given as Percentages

	Spleen beads	Liver beads	Bone marrow beads
Thymus suspension	57.5	147.4	17.8
	87.4	151.0	60.0
	117.3	154.6	59.8
	30.6	85.5	77.8
	–	139.3	51.6
	117.3	155.0	111.9
	63.1	98.9	45.4
	50.4	208.9	40.0
	58.6	100.0	62.6
Mean	72.8	137.8	58.5
Lymph gland suspension	71.8	153.7	36.2
	39.8	149.3	43.3
	55.0	153.2	38.1
	141.9	138.0	118.8
	77.2	140.7	54.1
	93.7	129.4	28.6
	54.9	126.8	57.0
	59.2	268.9	49.2
	32.9	77.6	48.7
Mean	69.6	148.6	52.7

tissue washed in Earl's solution their surfaces were carefully freed of remaining connective tissue and they were then washed again in Earl's solution The organs were then cut up finely and placed into a glass vessel 6 ml of Earl's solution were added and the pieces of tissue were stirred vigorously with a forceps or scissor blades and pressed against the sides of the glass vessel The tissue mixture was then sieved through ordinary muslin

2 ml of the thymus suspension and lymph gland suspension respectively were poured into each of the burettes with spleen liver and bone marrow beads and were allowed to pass through 5 5 cc of these after which the tap was closed 1 minute after the start the tap was opened and the suspension was allowed to pass through a further 0 5 cc of beads, after which the tap was closed again Every succeeding minute the suspension was passed through 0 5 cc of 'new' beads in this way At 9 minutes after the start the suspension was let down to the 10 cc mark and at 10 minutes it was released through the tap and the first three drops taken for examination The number of cells per cmm in the suspension before and after its passage through the beads vas then counted (dilution 1 20 or 1 100 2 × 32 B squares counted in the Burker chamber) The number per cmm after the passage was given as a percentage of the number per cmm beofre Three thymus suspensions and three lymph gland suspensions were passed through glass beads coated with saline extracts and four suspensions of each kind through glass beads coated with water extracts

RESULTS

As can be seen in Table 1, there were fewer cells per cmm after passage through spleen and bone marrow beads On the other hand there were more cells per cmm after passage through liver beads No significant difference were observed between the thymus and lymph gland cells

TABLE 1

No of Cells per cmm after the Passage through Glass Beads Coated with Extract as a Percentage of the Number per cmm before Passage

	Spleen beads	Liver beads	Bone marrow beads
Saline extract			
Thymus suspension	90 2	96 3	72 0
	103 9	182 4	68 6
	76 4	156 9	44 4
Mean	90 2	145 2	61 7
Lymph gland suspension	88 2	163 2	111 8
	93 2	106 8	50 0
	101 7	230 5	74 6
Mean	94 4	166 8	78 8
Water extract			
Thymus suspension	48 9	118 9	91 1
	56 1	129 0	61 3
	61 9	228 6	66 6
	88 2	202 0	80 4
Mean	63 8	169 6	74 9
Lymph gland suspension	44 9	146 9	67 3
	45 5	236 4	36 4
	51 3	200 0	74 4
	58 3	291 7	83 3
Mean	50 0	218 8	65 4

Experiment 2 B

Assume that aggregates occur in the cell suspensions and that these aggregates are of such an order of magnitude that a sample from the suspension taken with a fine pipette will contain fewer cells per cmm than a sample taken with a coarser pipette In this case the effect of the liver factor would be less noticeable if coarser pipettes were used for the taking of the samples In the following experiment which is a repetiton of experiment 2 A pipettes of different sizes were used

METHOD

0 2 ml of cell suspension was mixed with 0 2 ml of Earl s solution or with 0.2 of water extract of liver The number of cells per cmm was then counted in a Burker chamber after diluting 1 20 with Earl s solution The samples were taken 5 minutes after mixing both with so called blood pipettes of 0 025 cmm and with coarser pipettes of 0 05 or 0 1 cmm

RESULTS

Table 4 shows that in all cases a higher number of cells per cmm was obtained in the mixture with Earl s solution when the sample was taken with a coarser pipette Further the effect of the liver factor appeared to be less in all cases where a coarse pipette was used than in cases where finer ones were used An attempt at making a direct estimate of the aggregation tendency failed since in all mixtures this was very insignificant

TABLE 4

The Number of Cells per cmm after the Addition of Earl s Solution and of Liver Extract using Pipettes of Different Sizes for the Sampling

	0 025 + 0 475		0 050 + 0 950
A Thymus suspension + Earl s solution	4600	<	9400
B Thymus suspension + liver extract	19300		16300
B/A × 100	420	>	173
A Lymph gland suspension + Earl s solution	10300	<	12800
B Lymph gland suspension + liver extract	18600		18800
B/A × 100	181	>	147

	0 025 + 0 475		0 100 + 1 900
A Thymus suspension + Earl s solution	1350	<	2350
B Thymus suspension + liver extract	3700		3900
B/A × 100	274	>	166
A Lymph gland suspension + Earl s solution	2150	<	3750
B Lymph gland suspension + liver extract	4500		5600
B/A × 100	209	>	149

The error of the method in all of these investigations is very large, since relatively few cells were counted in the Burker counting chamber The coefficient of variation on counting 2 × 32 B squares from different samples of the same suspension is approximately 10 per cent The

When Earl's solution alone was passed through the liver beads in the same way as the cell suspensions, no cells were found in the fluid collected. Neither did direct examination of the liver extract reveal any cells. Thus it must be concluded that only thymus or lymph gland cells were counted after passage.

It is theoretically possible that a true increase in the number of cells occurs, but it is more probable that this is only apparent. The small number of cell aggregates, as a rule comprising fewer than 10 cells, which was observed in the suspensions on careful examination, may have been dispersed by some factor in the liver extract, so that on counting in the Burker chamber more cells were recorded than before the passage through the liver beads.

THE INFLUENCE OF LIVER EXTRACT ON THE NUMBER OF SUSPENDED LYMPHOID CELLS CONVENTIONALLY COUNTED

Experiment 2 A.

The following experment was carried out in order to preclude, if possible, a true cell increase as an explanation for the results.

METHOD

0.5 ml of Earl's solution was mixed with 0.5 ml of cell suspension (thymus and lymph gland suspensions prepared as in experiment 1). 0.5 ml of the same suspension was mixed with 0.5 ml of a water extract of liver. The number of cells per cmm was then counted in a Burker chamber (dilution 1·20, 2 × 32 B squares counted), both immediately after and also 20 minutes after mixing.

RESULTS

It may be seen in Table 3 that the number of cells per cmm after the addition of liver extract was more than 200 per cent of the number per cmm after the addition of Earl's solution. This effect of the liver extract occurred immediately. When the liver factor was added under the microscope no changes were noted among the cells. Further examination of the liver extract revealed no cells. The conclusion must therefore be drawn that the cell increase after the addition of liver extract was apparent.

TABLE 3

No. of Cells per cmm after the Addition of Earl's Solution and of Liver Extract

	Immediately	After 20 mins
A Thymus suspension + Earl's solution	8600	11300
B Thymus suspension + liver extract	30000	26200
A Lymph gland suspension + Earl's solution	3500	2200
B Lymph gland suspension + liver extract	10100	11500

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The Department of Pathology Section II University of Helsinki and from the Departments of Forensic Medicine, University of Helsinki and University of Turku Turku Finland

HISTOCHEMICAL OBSERVATIONS ON WOUND HEALING IN DENERVATED AND HEALTHY RAT SKIN

By

J RAEKALLIO, R LINDFORS, G ELFVING, J HÄSTBACKA and J PUITTINEN

Received 13 iii 64

Wound healing has been shown to be distinctly slower in denervated rat skin than in a healthy animal (*Elfving* 1959) Attempts to outline the pathogenesis of this retardation would be important both pathologically and surgically

In several previous investigations (*Raekallio* 1960, 1961, 1963 a, b, *Raekallio & Levonen* 1963 a b, c, *Raekallio* 1964, *Raekallio & Seligman* 1964) histochemical methods for enzymes have been demonstrated to provide a tool for detecting some of the very first reactions in healing wounds The differential enzymatic response to injury in denervated and healthy skin is the particular object of this investigation

MATERIAL AND METHODS

12 healthy four month old Sprague Dawley rats of approximately 200–250 g weight were used All the animals were kept on standard laboratory [illegible]

[illegible] nerve trunks The surgical procedure caused unilateral denervation of a skin zone to the left The animals displayed paralysis and flaccidity of the abdominal muscles causing a characteristic bulging of the left side of the abdomen

Two weeks postoperatively circular skin wounds 5 mm i diameter were cut with sharp earmark scissors on the hypaesthetic denervated side where the abdominal bulge was most pronounced No anaesthesia was used The contralateral intact side was similarly wounded at the corresponding site Thus each animal served as its own control Immediately before the operation the area was washed and shaved The skin inside the circles was excised with fine scissors Neither ligatures nor sutures were used

Four a[illegible] intervals [illegible] taining [illegible] was fro[illegible] [illegible] nail was fixed overnight in

[illegible]

results from the different experiments agree so well, however, that they are considered worthy of publication The observations have also been confirmed in a larger unpublished study by *Stillstrom* He found a statistically significant scattering effect of the same type of liver extract in a concentration of 0 05 The effect of the extract was destroyed by heating to 60° C There was no similar effect of the bile The serum showed a less potent (but statistically significant) effect than the liver extract

We have not learned much about this "liver factor" from these experiments, but they have shown that considerable errors are involved in calculations of the number of cells in artificial suspensions of lymphoid cells The adding of liver extract may be a useful method by which to obtain the correct cell number and to estimate the degree of aggregation in such suspensions

SUMMARY

The number of cells per ccm in suspensions of thymus and lymph gland cells increased after passage through glass beads coated with water or saline extracts of liver (experiment 1 A) The total number of cells in the suspensions also appeared to increase on passage through these "liver beads" (experiment 1 B) The addition of cell-free liver extract to thymus and lymph gland suspensions increased the number of suspended cells (experiment 2 A) The cell increase occurred immediately and, as far as could be judged, was only apparent Experiments with pipettes of different diameters support the view that the liver factor contributes to the dissolution of cell aggregates in the suspensions (experiment 2 B)

The addition of liver extract may be a useful method by which to obtain the correct cell number in suspensions of lymphoid cells and to estimate the degree of aggregation in such suspensions

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Stillström J Unpublished results



Figs 1–2

Fig 1 Aminopeptidase activity in the peripheral zone of a healthy rat skin wound age of the lesion 24 hrs × 100

Fig 2 Aminopeptidase activity in the peripheral zone of a denervated rat sk wound age of the lesion 24 hrs × 100

cold (+4° C) neutral buffered 10 per cent formalin The fixed specimens were further processed in the conventional way for paraffin sectioning and staining by the *van Gieson* method

In the fresh frozen halves aminopeptidase activity was visualized by the method of Nachlas et al (1957), alkaline phosphatase by the technique of *Grogg & Pearse* (1952), nonspecific esterase by the α naphthyl acetate modification as described by *Pearse* (1960), and cytochrome oxidase by the method of *Burstone* (1959) The 15 μ sections cut in a cryostat, were fixed for 5 minutes in cold neutral 10 per cent formalin before the demonstration of phosphatase and esterase activities By contrast, aminopeptidase and cytochrome oxidase were visualized without any previous fixation

RESULTS

Two zones could be noticed around the wounds In the immediate vicinity of the wound edge, a central or superficial zone, 200 to 500 μ in depth, showed decreasing enzymatic activity in the connective tissue cells In this area, signs of necrosis were histologically demonstrable in 24-hour and 96-hour wounds

Surrounding the central area, a 100 to 300 μ deep peripheral zone exhibited an increase in the enzymatic activity, initially in local fibroblasts Additionally, in 4-hour wounds polymorphonuclear leucocytes began to immigrate the peripheral zone, contributing thus to the increased enzymatic activity These cells were largely superseded by mononuclear cells in 24-hour and 96-hour wounds

An increase in the activity of all of the four enzymes studied was noticed in the peripheral zone, following four hours after the injury This zone reached a maximal stainability in 24-hour wounds, and the enzymatic activity remained strong also in 96 hour wounds

The difference between denervated and control wounds of each animal was first seen in the enzymatic response to injury In 4-hour wounds the activity of aminopeptidase and esterase was much less increased in the peripheral zone of the denervated wounds than in the control ones The difference became still clearer in 24-hour wounds (Figs 1 and 2), and later on The activation of alkaline phosphatase and cytochrome oxidase showed a similar retardation in the peripheral zones of the denervated wounds, especially from 24 to 96 hours after the injury

There was an additional difference between the two types of wounds also the granulocytic response to injury was distinctly retarded after denervation In 4-hour denervated wounds immigrating polymorphonuclear leucocytes rarely occurred in the peripheral zone of the lesions (Fig 4) By contrast, these cells were apparently more numerous in that zone of 4-hour control wounds Fig 3) The difference became less conspicuous in 24-hour wounds, and was hardly visible 96 hours after the injury

DISCUSSION

Loss of enzyme activity in the central zone apparently represents an early sign of imminent necrosis (*Raekallio* 1961, *Raekallio & Levonen*

1963 a, *Raekallio & Seligman* 1964) Later on, the necrosis could be demonstrated also histologically

The initial increase in enzyme activity of the peripheral zone seems to represent an adaptive defence mechanism (*Raekallio* 1960, 1961, *Raekallio & Seligman* 1964) by the local connective tissue cells, as a response to the injury Later on, the immigrating leucocytes significantly contribute to the intensification of the enzyme activity

In denervated skin wounds, the enzymatic response to injury was retarted and less intense than normally

Considering esterases, these enzymes are known to be present in large amounts in cells engaged in histiocytic function (*Braunstein et al* 1958) The increase in esterase activity observed in the connective tissue cells in healing wounds is apparently indicative of a change in their function towards that of phagocytosis (*Raekallio & Levonen* 1963 b) In the denervated skin wounds the phagocytic ability of cells thus seems to be less pronounced than normally

Aminopeptidase activity has been related to the proliferation of the connective tissue cells (*Monis et al* 1959) In addition, this enzyme could bear a correlation to certain peptides (*Raekallio* 1961) which are known to give rise to local hyperaemia, increased capillary permeability and migration of leucocytes The last mentioned possible effect of this enzyme might be connected with the retardated granulocytic response observed in denervated wounds which simultaneously exhibit a less pronounced increase in aminopeptidase activity The weak leucocytic response, on the other hand, explains, in part, the relatively slow healing of the denervated wounds As is well known, these cells have several beneficial properties in the process of healing

Several theories exist concerning the possible functions of alkaline phosphatase *in vivo* (cf *Raekallio* 1961) *E g Dunphy* (1959) related the activity of this enzyme in the wound to the general phenomenon of cell proliferation In fact, recent biochemical and biophysical investigations (cf *Lindner* 1962) have shown signs of proliferation in connective tissue cells, when examined 16 to 32 hours after the injury

Cytochrome oxidase participates in the respiratory processes of cells Aerobic oxidation is necessary for the occurrence of mitoses (*Bullough & Johnson* 1951) Also the activity of cytochrome oxidase, as an indicator of oxidation, was less conspicuous in the denervated wounds

Thus, by using all the four histochemical methods, the enzymatic response to injury was weaker in the denervated than in the control wounds These experiments have pointed out one possible way, enzyme histochemistry, to investigate further the complicated but interesting problem of the delayed healing of denervated wounds

SUMMARY

The differential enzymatic response to injury in denervated and healthy skin was experimentally investigated by using 12 albino rats





Figs 3 4

Fig 3 Cellular reactions in the peripheral zone of a healthy 4 hour skin wound × 100

Fig 4 Small amount of invading leucocytes in the periphery of a denervated 4 hour skin wound × 100

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The Departments of Pathology II (Head. S Falkmer) and Clinical Bacteriology (Head S Bergman), University of Umeå, Umeå, Sweden

EXPERIMENTAL ABSCESSES IN BRAIN AND SUBCUTIS

A Microangiographic Study in the Rabbit

By

O HASSLER and A FORSGREN

Received 27 ii 64

During the first decades of this century several experimental studies on cerebral abscesses appeared Some of the most important investigations were those carried out by *Homen* (1912), *Groff* (1934), and *Falconer, McFarlan & Russell* (1943)

The vascular reaction plays an important rôle in abscess demarcation and healing The purpose of this investigation is to analyse the vascular response in the neighbourhood of cerebral and subcutaneous abscesses

MATERIAL

Adult rabbits were used The material was divided into a main series and an extra series The main series comprised about fifty rabbits but several of these died of septicaemia irrespective of the kind of bacteria during the first days after the operation These rabbits were excluded from the study Consequently, the main series comprised 29 surviving rabbits (see Table 1) The extra series contained 10 animals (see Bacteriological methods)

METHODS

Surgical Technique

The [illegible]

[illegible] a subcutaneous injection of 0 05 ml of the same agar suspension of bacteria This injection was made deep into the subcutis of one leg

Bacteriological Methods

The nutrient agar was prepared according to *Falconer, McFarlan & Russell* (1943) Thus one bacterial colony was taken from a pure culture on an agar plate It was inoculated in a broth tube and the bacteria were allowed to grow for 24 hours at 37° C One drop of [illegible] gar solution The following [illegible] *Proteus vulgaris* Neisseria [illegible] *faecalis*

When the main s[illegible] the abscesses varied somewhat and we suspected that *Falconer, McFarlan & Russell*'s

Denervation was produced by gangliectomy on one side of the animals, the other side of each rat served as a control Two weeks postoperatively circular skin wounds were excised on both sides Aminopeptidase, alkaline phosphatase, esterase, and cytochrome oxidase activity was demonstrated both in the denervated and in the control wounds The specimens were investigated also histologically By means of all the four histochemical methods, the increase in the enzymatic activity of the peripheral wound zone was retardated and less pronounced in the denervated wounds than in the controls In addition, a relatively scant immigration of leucocytes characterized the denervated wounds Thus, both the enzymatic and the granulocytic response to injury was weaker in the denervated than in the healthy skin wounds

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MATERIAL

Adult rabbits were used The material was divided into a main series and an extra series The main series comprised about fifty rabbits but several of these died of septicaemia irrespective of the kind of bacteria during the first days after the operation These rabbits were excluded from the study Consequently, the main series comprised 29 surviving rabbits (see Table 1) The extra series contained 10 animals (see Bacteriological methods)

METHODS

Surgical Technique

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Th [illegible]
al [illegible]
ce [illegible] ...ct 0 05 ml of the same agar suspension of bacteria This injection was made deep into the subcutis of one leg

Bacteriological Methods

The nutrient agar was prepared according to *Falconer McFarlan & Russell* (1943) Thus one bacterial colony was taken from a pure culture on an agar plate It was inoculated in a broth tube and the bacteria were allowed to grow for 24 hours at 37° C One drop of the broth culture was added to 1 ml of the nutrient agar solution The following types of bacteria were used *Staphylococcus aureus Proteus vulgaris Neisseria flavescens Streptococcus pyogenes* and *Streptococcus faecalis*

When the main series had been investigated we found that the size of the abscesses varied somewhat and we suspected that *Falconer McFarlan & Russell's*

TABLE 1

Survey of the Material (Main Series)

Rabbit no	Type of bacteria	Cerebral injection	Subcutaneous injection	Killed after (days)	Investigation	Cranial abscess	Subcutaneous abscess
1	Staph. aur.	+	—	4	peroxid.	intracerebr.	—
2		+	—	4			—
3		+	+	5			+
4		+	+	5	microrad.		+
5		+	—	6	peroxid.		—
6		+	—	8			—
7		+	—	9			—
8		+	+	10			+
9		+	+	10			+
10		+	+	11	microrad.		+
11		+	—	14			—
12		+	—	15			—
13		+	+	15	peroxid.		+
14		+	+	16			+
15		+	+	18	microrad.	subdural	no abscess
16		+	—	19		intracerebr.	—
17		+	+	20		subdural	+
18		+	—	20	peroxid.	intracerebr.	—
19		+	+	21	microrad.	epidural	no abscess
20	B. coli	+	+	10	peroxid.	no abscess	+
21		+	+	10		intracerebr.	+
22	Str. faec.	+	+	5			+
23		+	+	5			+
24		+	+	8			+
25	Str. zym.	+	+	8			+
26		+	+	8			+
27	Proteus	+	+	10			+
28	Neiss. flav.	+	+	10			+
29		+	+	10			+

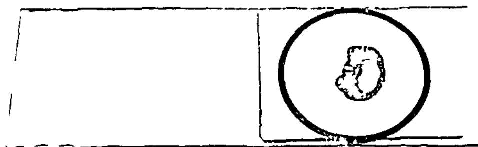


Fig. 1

A brass ring 0.2 mm high lies between the glass slide and the cover slip. The hole (Canada balsam) between the two sections can be removed, embedded and be stained by the routine stains (hematoxylin eosin).

method included a factor of some inexactness, since the number of bacteria injected was not determined, and bacteria may grow more or less rapidly in a broth owing to various factors which are not easily controlled Therefore, 10 additional rabbits were inoculated with a known number of bacteria Two animals received 50 milliard, two 5 milliard, two 500 million and two others 50 million *Streptococci faecalis* by each injection of 0 05 ml of the nutrient agar solution Two other animals received sterile agar with standard broth

Specimens were taken for culture from 7 abscesses (two subcutaneous and two cerebral abscesses caused by *Staphylococcus aureus* and further, one subcutaneous and two cerebral abscesses caused by *Neisseria flavescens*) The bacteria were identified by bacteriological techniques and were found to be the same as those injected

Microangiographic Methods

The vasc lar react ons are nd the abc

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the microradiographic method excellent stereoscopic pictures are obtained of large blood vessels After angiographic examination, both methods permit the specimens

body and freed from skin, muscles jaws ears and eyes The cranial cavity was opened frontally, so that the olfactory bulbs were exposed The cran m with the

place

thick

for more than a few days)

contrast medium used was an aqueous similarly inserted in the abdominal pared according

jection started

mm Hg The

same way as previously described for peroxidase staining. After fixation the specimens were cut in a freeze microtome into slices 400–2000 μ thick. The X-ray examination was performed with the aid of a Philips diffraction tube (25633/6?) with an extremely fine focus (0.16mm²). The radiation was generated at 25 kV and 30 mA and allowed to pass through a Ni filter. The film focus distance was 100 cm. The slices were stored in thin walled plastic bags during the X-ray investigation in order to prevent drying. Gevaert Scientia 5F56 or Kodak Maximum Resolution plates were used. In order to obtain a stereoscopic picture of the vascular tree the examination of the microradiograms was carried out according to *Bellman* (1953).

Histological Methods

A specimen from each of the abscesses microradiographically examined and from one of the abscesses examined with the peroxidase method were embedded in paraffin, cut in 10 μ thick sections and stained with the methods of van Gieson or Nissl (*Romeis* (1948) modification for formalinfixed material).

RESULTS

Main Series

Intracranial abscesses were obtained in all but one of the 29 rabbits. Most of the intracerebral abscesses were round or ovoid, their maximum diameters varied between 2 and 9 mm.

The vascular reaction around the cerebral abscesses was very weak. After 4–8 days a zone, less than 1 mm wide, of solitary dilated vessels was observed around the abscess in the peroxidase stained sections. In addition there was a diffuse oedema around the abscess (Fig. 2).

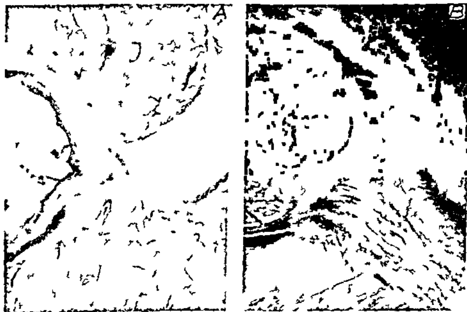


Fig. 2

Peroxidase stained frozen section through abscess 8 days old. *A* Cerebral abscess. Note the slight hyperaemia and the spongious oedema around the abscess (to the left) × 15. *B* Subcortical abscess from the same rabbit. A distinct zone of dilated vessels (stained black) is seen at the margin of the abscess × 15.



Fig 3

Microradiograms of slices 1 mm thick *A* Cerebral abscess, 14 days old × 2 5 *B* Subcutaneous abscess 14 days old × 2 5 *C* and *D* High magnifications of the areas of newly formed vessels in *A* and *B* *E* and *F* Histological sections through the same slices stained with Nissl s and van Gieson's methods respectively, × 30

After 9 11 days, there occurred in the thin capsule of the intracerebral abscesses solitary, delicate vessels, which had an anomalous course and evidently were newly-formed After 14–16 days the zone of newly-formed vessels was less than 1 mm thick (Fig 3). However, the zone

same way as previously described fr... n the specimens were cut in a freeze microto... e X-ray examination was performed with the ... 333/62) with an extremely fine focus (0 16mm²). ... and 30 mA and allowed to pass through a Ni-f... 100 cm. The slices ... to pre... used ... of the microradiograms was carried out according to *Bellman* (1953).

Histological Methods

A specimen from each of the abscesses microradiographically examined, and from one of the abscesses examined with the peroxidase method were embedded in paraffin, cut in 10-μ-thick sections and stained with the methods of van Gieson or Nissl (*Romeis* (1948) modification for formalinfixed material).

RESULTS

Main Series.

Intracranial abscesses were obtained in all but one of the 29 rabbits. Most of the intracerebral abscesses were round or ovoid; their maximum diameters varied between 2 and 9 mm.

The vascular reaction around the cerebral abscesses was very weak After 4–8 days a zone, less than 1 mm wide, of solitary dilated vessels was observed around the abscess in the peroxidase-stained sections. In addition there was a diffuse oedema around the abscess (Fig. 2).



Fig 2.

Peroxidase-stained, frozen sections through abscesses, 8 days old *A* Cerebral abscess. Note the slight hyperaemia and the spongious oedema around the abscess (to the left). × 15. *B*. Subcutaneous abscess from the same rabbit A distinct zone of dilated vessels (stained black) is seen at the margin of the abscess × 15.

of the subcutaneous abscesses Especially in the striated muscles which bordered on these abscesses hyperaemia was conspicuous In the capsule of subcutaneous abscesses that were 6 days old or above several newly formed vessels were noted These vessels were more frequent and of a coarser calibre in the subcutis than in the brain After 18 21 days the abscesses as well as the hyperaemia and the newly formed vessels showed regression No differences were observed between abscesses caused by various types of bacteria

Histological sections through the abscesses showed a central core of pus surrounded by tissue heavily infiltrated with polymorphonuclear granulocytes As is usually seen in brain abscesses there was a thin *fibroblastic capsule and peripheral to it a moderate gliosis* and a diffuse oedema The capsule was a little thicker in the cortex than in the white matter The capsule was more developed in the subcutaneous abscesses The impressed avascular parts of the brains bordering on subdural or epidural abscesses showed a disappearance of nerve cells and gliosis (Fig 4 B) The newly formed vessels occurred in the capsule They had very thin walls not only in the brain but also in the subcutis Their walls were rich in fibroblasts

Results in the Extra Series

In the two rabbits which received sterile agar no abscesses could be detected at autopsy 5 days after the injections Of the 8 rabbits which had received *Streptococcus zymogenes* in various concentrations 3 died of septicaemia during the first days after injection These three rabbits had received a dose of 50 milliard 5 milliard and 500 million bacteria respectively

The surviving rabbit which had been given 50 milliard bacteria had very large abscesses both in the brain and in the subcutis The cerebral abscess was rounded and about 10 mm in its largest diameter the subcutaneous abscess was elongated (about 20 × 4 × 4 mm)

The surviving rabbit that had received 5 million bacteria had only small abscesses the size of a pin s head The rabbit receiving 500 million bacteria had rounded abscesses which were about 5 mm in their largest diameter whereas the two rabbits which received 50 million bacteria had still smaller abscesses (about 3–4 mm in their largest diameter)

DISCUSSION

We have not been able to find any previous report in the literature on the difference between brain and subcutis in the vascular reaction around abscesses with the exception of *Greenfield s* (1963) He states that the capsule is not so well developed in the brain as in other organs Vascular reactions around abscesses have certainly not been studied with the new microangiographical methods which have been developed

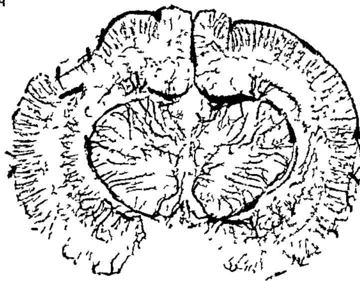




Fig 4

A Microradiogram from the animal with a subdural abscess causing an impression (upwards to the left) of the brain parenchyma *B* A histological section through the impressed part of the same section Nissl's stain Magnification, × 4 and 30 respectively

was somewhat thicker and contained larger vessels in those parts of the margins of the abscesses, which faced the richly vascularized cerebral cortex and the large vessels at the base of the brain, than in other parts The hyperaemia was still minimal.

After 15 18 days, the number of dilated vessels was smaller and their degree of dilatation was reduced as compared with findings after the shorter periods of observation After 18 21 days, there was quite a definite regression of the intracerebral abscesses and of the vascular reaction around them Three abscesses, in animals killed after 18, 20 and 21 days, respectively, had probably discharged outside the brain, so that they were situated subdurally, or in one case, even epidurally These abscesses were encapsulated by thickened meninges The brain parenchyma in the neighbourhood was torn and impressed (Fig 4), corresponding to the abscess The impressed parts were poorly vascularized

Subcutaneous abscesses were produced in all but two of the rabbits which received a subcutaneous injection of bacterial agar These abscesses were of about the same size as the cerebral abscesses They exerted a stronger vascular reaction than the latter The zone of dilated vessels around the abscess was slightly thicker in the subcutaneous than in the cerebral abscesses There was a larger number of dilated vessels, with a greater degree of dilatation, around the subcutaneous abscesses than around the cerebral abscesses The most pronounced hyperaemia was generally observed in the deep and proximal parts of the margins

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during recent years and which afford several advantages over the classical methods (*Bellman* 1953, *Gothman* 1961, *Ljungqvist* 1963) In other kinds of inflammation, *e g* in cryptococcosis, it is also observed that the vascular reaction is much weaker in the brain than in other organs (*Bergman* 1961, *Bergman & Hassler,* to be published) We have also frequently noted that there is practically no vascular reaction around the common granulomata of Encephalitozoonosis cuniculi in the rabbit brain It is not easy to determine the cause of this weak reaction of the cerebral vascular system Within the cranium, however, space is limited and a vascular reaction, with hyperaemia and newly-formed vessels, will inevitably increase intracranial pressure and cause a compression of the remaining part of the brain Further, the white matter develops a pronounced oedema, which is generally considered to be the most conspicuous feature on the margins of the cerebral abscesses (*Wohlwill* 1959) This oedema may diminish the dilatation of the vessels

There are great differences of angioarchitecture in the cerebral cortex, the subcortical nuclei, and the white matter (*Lindgren* 1940, *Finley* 1940, *van den Bergh* 1961) Capillarization is much richer in the two first-mentioned locations than in the last one In this investigation the vascular reaction to an abscess was found to be slightly more marked in the cortex than in most parts of the white matter This is in accordance with the fact that abscesses form much more commonly in the white than in the grey matter (*Greenfield* 1963)

Earlier investigators (*Falconer, McFarlan & Russell* 1943) observed only very slight morphological differences between cerebral abscesses caused by various kinds of bacteria Nor did we find any great differences Probably the number of bacteria in the agar injected had some, though not a great, importance, and sterile agar caused no abscesses Some of the types of bacteria, which we investigated, belong to those most commonly found in cerebral abscesses in man (*Falconer, McFarlan & Russell, Wohlwill* 1959)

SUMMARY

Abscesses were produced by injecting agar suspensions of various bacteria into the brains of 29 rabbits For comparison, subcutaneous abscesses were similarly induced in most of the animals The vascular reactions around the abscesses were studied with the aid of microangiographic methods The hyperaemic zone was narrower and less pronounced, and the newly formed vessels were thinner and less frequent in the brain than in the subcutis In the brain most of the newly-formed vessels were observed in such parts of the margins of the abscesses as faced the large basal arteries or the richly vascularized cerebral cortex Abscesses caused by various bacteria showed no angiographical differences

TABELLE

Vereinfachtes Antigen-Schema der Salmonella Sub-Genera II und III (1963)

O	H 1 Phase	H 2 Phase
B	b	—
B	e,n	l
B	G	—
B	G	e,n
B	L	e n
B	z	e n
B	z_{39}	l
C	a	z_{42}
C	a	z_{52}
C	b	e n
C	b	e n z_{42}
C	b	z_{42}
C	G	l
C	G	e n
C	k	z
C	L	l
C	L	z
C	L	Ar 25
C	z	l
C	z	z_{42}
C	z_4	—
C	z_{36}	—
C	z_{36}	z_{42}
C	z_{39}	l
C	z_{42}	l
C	z_{52}	Ar 25
D	b	e n
D	b	z_6
D	c	z_{39}
D	e n	l
D	G	—
D	G	e n
D	G	z_{42}
D	L	e n
D	z	l
D	z	e n
D	z	z_6
D	z	z_{39}
D	z_4	z_{35} z_{42}
D	z_{23}	l
D	z_{39}	l
F	e n	l
F	G	—
F	L	e n
F	L	z_4
F	z	l
F	z	e n
F	z_{38}	z_{42}
E	z_{39}	l
11	d(a)	d e n
11	G	—
11	G	e n
11	G	z_{39}
11	k	Ar 25
11	L	e n
11	z_4	—
13	b	z_{42}
13	G	—
13	G	l
13	G	e n
13	z	l
13	z_4	—
13	z_{29}	l
13	z_{29}	e n
13	z_{42}	l
16	e n	l
16	G	—
16	G	l
16	k	z
16	L	l
16	L	z
16	L	z_{35}
16	z_4	—
16	z_{10}	e n
16	z_{29}	—
16	z_{42}	l
17	b	e n
17	e n	l
17	G	—
17	G	e n
17	L	—
17	L	z_{35}
17	z_4	—
17	z_{10}	e n
17	z_{36}	—
18	b	e n
18	k	Ar 25
18	z_4	—
18	z_{10}	z
18	z_{10}	z_6
18	z_{10}	Ar 38
18	z_{36}	—
21	G	—
21	i	e n
21	L	z
21	z_4	—
21	z_{10}	z_6
21	z_{29}	—
28	b	z_6
28	z	z_{39}
30	G	—
35	G	—

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VEREINFACHTES ANTIGEN-SCHEMA DER SALMONELLA SUB-GENERA II UND III

Von

F. KAUFFMANN

Eingegangen 3. 1. 64

Im Jahre 1962 haben *F. Kauffmann* & *R. Rohde* in 2 Mitteilungen vorgeschlagen, die *species* der *Salmonella sub-genera* II und III (= Arizona) nur mit Hilfe des vereinfachten *Kauffmann-White-Schemas* zu diagnostizieren. Nachdem kürzlich *F. Kauffmann* & *A. Petersen* eine Tabelle mit vereinfachten *Salmonella sub-genus* II-Formeln (bis Ende 1962 gültig) publiziert haben, sind in der folgenden Tabelle die bis Ende 1963 festgestellten *species* der *sub-genera* II und III angegeben, ohne aber für die Vollständigkeit der Arizona-*species* zu garantieren. Es sei hervorgehoben, dass hierdurch die beiden *sub-genera*, die nahe mit einander verwandt sind, in einem einzigen Antigen-Schema (dem vereinfachten K.-W.-Schema) zusammengefasst sind. Ferner sei beachtet, dass bereits mehrere dieser vereinfachten Formeln im *sub-genus* I vorkommen. So entspricht zum Beispiel die Formel B: b: ... auch der vereinfachten Formel von *S. java* und B: G: ... auch der vereinfachten Formel von *S. derby* etc. Da aber die *species* des *sub-genus* I im allgemeinen nach dem originalen K.-W.-Schema diagnostiziert werden, spielt dieses in der Praxis keine Rolle. Um Missverständnisse zu vermeiden, sollte man B: G: ... (*sub-genus* II) schreiben, falls es sich in diesem Falle um eine *species* des *sub-genus* II handelt. In der Praxis dürfte es aber im allgemeinen genügen, dem einsendenden Arzte nur die Diagnose: *Salmonella sub-genus* II (Enteritis-Erreger) mitzuteilen. Bis zum Beweise des Gegenteils spielt die exakte Diagnose der *sub-genus* II *species* im allgemeinen keine praktische Rolle. Es ist deshalb überflüssig, derartige Kulturen nach dem originalen K.-W.-Schema zu diagnostizieren oder mit Eigennamen zu belegen.

O	H	
	1 Phase	2 Phase
48	k	e n
48	k	z_{35}
48	k	z_{39}
48	k	Ar 25
48	L	1
48	L	1 z_{47}
48	r	e n
48	z_{4}	—
48	z_{10}	1
48	z_{10}	e n
48	z_{29}	—
48	z_{38}	—
48	z_{29}	z
48	z_{29}	z_{35}
50	e n	1
50	G	—
50	G	1
50	i	1
50	i	z
50	k	1
50	k	e n
50	k	z
50	k	z_{35}
50	L	z_{45}
50	r	1
50	r	z
50	z	e n
50	z_{4}	—
50	z_{10}	—
50	z_{10}	z
50	z_{10}	z z_{4}
50	z_{9}	—
50	z_{36}	—
50	z_{29}	1
50	z_{39}	z
50	z_{29}	z_{3}
50	z_{3}	Ar 25
51	G	—
51	z_{4}	—
51	z	z
51	—	1
52	l	Ar 25
52	—	1
53	G	—
53	i	z
53	k	e n
53	k	z_{3}
53	l	z_{39}
53	r	z
53	r	Ar 25
53	r	Ar 38
53	z	1
53	z_{4}	—
53	z_{29}	Ar 25
55	k	z_{39}
56	b	—
56	z_{4}	—
57	i	e n
57	z_{9}	z_{40}
57	z_{39}	e n
58	l	1
58	L	e n
58	L	z_{35}
58	r	e n
58	r	Ar 25
58	r	Ar 25 z_{4}
58	z_{29}	z
59	k	(z)
59	k	z_{35}
59	k	Ar 25
59	z_{4}	—
59	z_{10}	Ar 25
59	z_{10}	Ar 40
59	z_{9}	—
59	z_{29}	—
60	k	z
60	k	Ar 25
60	L	z
60	r	e n
60	r	z
60	z	e n
60	z_{10}	z
60	z_{29}	Ar 25
61	c	1
61	c	z_{3}
61	i	z
61	i	Ar 25
61	k	—
61	k	1
61	L	1
61	L	z_{3}
61	r	1
61	r	Ar 25
61	z_{29}	Ar 25
Ar 6	G	—
Ar 6	z_{4}	—
Ar 8	G	—
Ar 8	z_{4}	—
Ar 8	z_{36}	—
Ar 29	c	z
Ar 29	i	z
Ar 29	i	z_{35}
Ar 29	i	Ar 40
Ar 29	k	1

O	H 1 Phase	H 2 Phase
35	i	—
35	i	en
35	i	z
35	k	z
35	k	Ar 25
35	L	l
35	L	z_{35}
35	r	en
35	r	z_{35}
35	r	Ar 41
35	z_4	—
35	z_{10}	z_3
35	z_9	—
35	z_5	l
35	z_5	en
38	d	—
38	G	—
38	i	Ar 25
38	l	z
38	l	z_3
38	k	Ar 34
38	k	Ar 37
38	L	z_{35}
38	L	Ar 25
38	I	Ar 34
38	r	z
38	r	Ar 25 z_{17}
38	z_4	—
38	z_{10}	z
38	z_{10}	Ar 25
38	z_4	Ar 25
39	L	en
40	a	z_3
40	c	en
40	G	—
40	G	en
40	i	l
40	I	Ar 25
40	z	l
40	z_4	—
40	z_{35}	en
40	z_3	—
40	z_{39}	l
41	G	—
41	k	—
41	z_4	—
41	z_{10}	l
41	z_1	z
41	z_9	—
41	z	—
42	b	l
42	b	en
42	G	—
42	k	z
42	I	l
42	L	en
42	r	—
42	r	Ar 25
42	z	l
42	z	en
42	z_4	—
42	z_{10}	en
42	z_{10}	z
43	en	l
43	G	—
43	G	z_4
43	I	Ar 25
43	z_4	—
43	z_{30}	—
43	z_4	—
43	z_4	l
44	G	—
44	z_4	—
44	z_{30}	—
45	a	en
45	a	z_{10}
45	G	—
45	G	l
45	G	en
45	z	z_{39}
45	z_4	—
45	z_9	—
47	a	—
47	b	I
47	c	en
47	d	z_{39}
47	i	z_3
47	i	Ar 25
47	I	l
47	l	l z_{50}
47	I	en
47	L	Ar 25
47	r	z
47	r	Ar 25
47	z	z
47	z_6	l
47	z_1	l
47	z_9	—
47	z	l
47	z	en
48	a	l
48	d	l
48	d	z
48	en	z
48	G	—
48	i	z_3 Ar 40
48	i	Ar 25
48	l	l

O	H	
	1 Phase	2 Phase
48	k	e n
48	k	z_{35}
48	k	z_{39}
48	k	Ar 25
48	L	1
48	L	1 z_{47}
48	r	e n
48	z_4	—
48	z_{10}	1
48	z_{10}	e n
48	z 9	—
48	z_{36}	—
48	z_{32}	z
48	z,°	z_{35}
50	e n	1
50	G	—
50	G	1
50	i	1
50	i	z
50	k	1
50	k	e n
50	k	z
50	k	z_3
50	L	z_{35}
50	r	1
50	r	z
50	z	e n
50	z_4	—
50	z_{10}	—
50	z_{10}	z
50	z_{10}	z z_{40}
50	z ɔ	—
50	z_{36}	—
50	z_{37}	1
50	z_{37}	z
50	z_{37}	z_{33}
ɔ0	z_{37}	Ar 25
51	G	—
51	z_4	—
51	z_{37}	z
51	—	1
52	L	Ar 25
5?	—	1
53	G	—
53	i	z
53	k	e n
53	k	z_{35}
53	1	z_{39}
53	r	z
53	r	Ar 25
53	r	Ar 38
53	z	1
53	z_4	—
53	z_{37}	Ar 25

O	H	
	1 Phase	2 Phase
5ↄ	k	z_{39}
56	b	—
56	z_4	—
57	ı	e n
57	z 9	z_{40}
57	z_{39}	e n
58	L	1
58	L	e n
58	L	z_3
58	r	e n
58	r	Ar 25
58	r	Ar 25 z_4
58	z,°	z
59	k	(z)
59	k	z_3
59	k	Ar 25
59	z_4	—
59	z_{10}	Ar 25
59	z_{10}	Ar 40
59	z 9	—
59	z,°	—
60	k	z
60	k	Ar 25
60	L	z
60	r	e n
60	r	z
60	z	e n
60	z_{10}	z
60	z ə	Ar 25
61	c	1
61	c	z_3
ſ1	ı	z
ſ1	ı	Ar 25
61	k	—
61	k	1
61	L	1
61	L	z_3
61	r	1
61	r	Ar 25
61	z,°	Ar 25
Ar 6	G	—
Ar 6	z_4	—
Ar 8	G	—
Ar 8	z_4	—
Ar 8	z_{36}	—
Ar 29	c	z
Ar 29	ı	z
Ar 29	ı	z_{35}
Ar 29	ı	Ar 40
Ar 29	k	1

O	H		O	H	
	1 Phase	2 Phase		1 Phase	2 Phase
Ar 29	L	z	Ar 30	k	Ar 25
Ar 29	z_{36}	—	Ar 30	l	z
			Ar 30	L	Ar 25
Ar 30	k	z	Ar 30	z_{10}	e n
Ar 30	k	z_{35}			

ZUSAMMENFASSUNG

Die vereinfachten Antigen-Formeln der beiden *Salmonella sub genera* II und III (= Arizona) werden in einer Tabelle, dem vereinfachten *Kauffmann-White-Schema*, angegeben.

LITERATUR

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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

6 *Examination of a Selection of Previously Untypable* H influenzae *M- and S-Strains for Evidence of Type Specific Antigens Different from the Used Type Reference Standards*

By

TOV OMLAND

Received 20 i 64

In several earlier studies (*Omland* 1963 a, b, d) a material of *H influenzae* strains has been examined for the occurrence of the known six type specific antigens Out of 163 strains about one third were shown to possess such antigens, a proportion which is in good agreement with other investigations Thus in the majority of strains no type specific antigens have been demonstrated According to the purpose and plan of the present series of studies (*Omland* 1963 a) it seemed logical as a next step to search for possible antigens of similar nature, but different from the known six type substances

MATERIAL AND METHODS

From the original material a selection was made of strains which on morphological grounds, seemed to offer the best possibilities in a search for such antigens The categories from which to select strains for study are illustrated in Table 1

TABLE 1

Distribution of H influenzae Strains According to Category of Variation and Serological Type as Determined by Gel Precipitation Tests

	Serological type						Total number typable	Number untypable	*Totals*
	a	b	c	d	e	f			
Mucoid	5	36	0	4	2	5	52	8	60
Smooth						2	2	100	102
Totals	5	36	0	4	2	7	54	108	162*

* The original material included also one R strain (total 163)

O	H		O	H	
	1 Phase	2 Phase		1 Phase	2 Phase
Ar 29	L	z	Ar 30	k	Ar 25
Ar 29	z_{36}	—	Ar 30	L	z
Ar 30	k	z	Ar 30	I	Ar 25
Ar 30	k	z_3	Ar 30	z_{10}	e n

ZUSAMMENFASSUNG

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In several earlier studies (*Omland* 1963 a, b, d) a material of *H. influenzae* strains has been examined for the occurrence of the known six type specific antigens. Out of 163 strains about one third were shown to possess such antigens, a proportion which is in good agreement with other investigations. Thus in the majority of strains no type specific antigens have been demonstrated. According to the purpose and plan of the present series of studies (*Omland* 1963 a) it seemed logical as a next step to search for possible antigens of similar nature but different from the known six type substances.

MATERIAL AND METHODS

From the original material a selection was made of strains which on morphological grounds seemed to offer the best possibilities in a search for such antigens. The categories from which to select strains for study are illustrated in Table 1.

TABLE 1

Distribution of H. influenzae Strains According to Category of Variation and Serological Type as Determined by Gel Precipitation Tests

	Serological type						Total number typable	Number untypable	Totals
	a	b	c	d	e	f			
Mucoid	5	36	0	4	2	5	52	8	60
Smooth						2	2	100	102
Totals	5	36	0	4	2	7	54	108	162*

* The original material included also one R strain (total 163).

O	H		O	H	
	1 Phase	2 Phase		1 Phase	2 Phase
Ar 29	I	z	Ar 30	k	Ar 25
Ar 29	z_{36}	—	Ar 30	I	z
			Ar 30	L	Ar 25
Ar 30	k	z	Ar 30	z_{10}	e n
Ar 30	k	z_3			

ZUSAMMENFASSUNG

Die vereinfachten Antigen Formeln der beiden *Salmonella sub genera* II und III (= Arizona) werden in einer Tabelle, dem vereinfachten *Kauffmann-White Schema,* angegeben

LITERATUR

Kauffmann F & Petersen A Über das ... ella Sub Genus II Species Acta pat ...
Kauffmann F & Rohde R Eine Verc ... gnose Acta path et microbiol scandinav 54 473–478 1962
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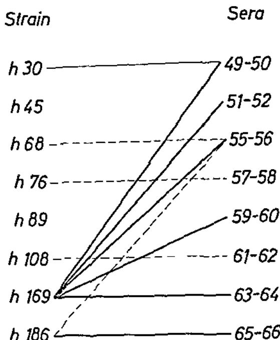


Fig 1
Homologous (horizontal lines) and heterologous (oblique lines) reactions between strains selected for examination and corresponding antisera
Broad line strong reaction
Narrow line moderate reaction
Dotted line weak reaction

series of gel precipitation tests was carried out in order to detect eventual subdivisions within that type At first the whole material of 163 H influenzae strains (*Omland* 1963 a) was set up against the set of sera provided by dr *Leidy* In addition to the e-strains already detected, the strains h 129, h 131, and h 145 (see Table 2) were found to be type e strains An extra control was obtained by testing the sera produced by means of the strains under examination (see Fig 1) against type reference antigens (*Omland* 1963 d), including a reference antigen prepared in the described manner from strain h 186 and from a type e strain provided by dr *Leidy* (designed e Mo) These investigations verified that strain h 186 belonged to type e

It thus appeared that two modifications of type e had been demonstrated, represented by the strains e 51 and h 186 Based upon these findings a comparison was set up of the two e-systems in gel precipitation The result is shown in Fig 2

According to Fig 2 it may be assumed that the type specific e antigens as prepared by the modified *MacPherson*'s procedure contain 3 factors (indicated by roman numerals), of which h 186 possesses main-

The 8 mucoid strains untypable by gel precipitation deserved obviously a closer investigation In addition 9 smooth strains with especially shiny colonial appearance were chosen for study In Table 2 a review is presented of the strains subjected to examination Additional studies were performed on strains related to type e and f

TABLE 2
H influenzae *Strains Previously Untypable by Gel Precipitation Selected for Further Examination*

	Capsular swelling untypable		Capsular swelling typable (type e?)		Totals
	Strain designations	Number	Strain designations	Number	
Mucoid	h 30, h 45, h 108 h 186 h 195	5	h 129, h 131 h 145	3	8
Smooth	h 4, h 6 h 10 h 16 h 17, h 68 h 76 h 89 h 169	9			9
Totals		14		3	17

Rabbits were inoculated with live antigens prepared from each of the listed strains with the exception of h 129, h 131 and h 145, which formed a particular group because of the results of capsular swelling typings, indicating a possible connection with type e Two animals were inoculated with each antigen, following the procedure described earlier (*Omland* 1963 b), however with a reduced number of inoculations (8–10) This was done in an attempt to avoid too much antibody against antigens of non bacterial origin (*e g* components of the media) (*Omland* 1963 c d) Smaller volumes (0 10 ml) were also injected each time so that the total amount of antigen injected in any one animal was only about 1 ml

Gel precipitations (miniature method, *Omland* 1963 c) were set up with the purpose of trying out all sera against all antigens as prepared by the modified *MacPherson's* method (*Omland* 1963 d)

RESULTS

The results of the gel precipitations in the various homologous and heterologous combinations are presented in a simplified manner in Fig 1 For practical reasons each pair of sera produced by immunization with the same antigen were employed in mixture (equal parts) Only strains or sera yielding a reaction have been included in Fig 1

The occurrence of partly very strong heterologous reactions was a general feature With one exception (see below), the homologous reactions were never stronger than the heterologous ones This fact speaks in itself against the occurrence among these strains of substances of type specific nature

The exception was strain h 186 which gave very strong precipitation against its homologous antisera As it was established also on repeated tests, that antigen from the said strain did not yield precipitation against any of the previously used type sera, it was tried against a set of type sera kindly provided by dr *Grace Leidy*, New York The type e serum from this set yielded a strong precipitation against h 186

These examinations indicated heterogeneity within type e, and a

DISCUSSION

The occurrence among the 17 strains subject to investigation in this study, of generally stronger heterologous than homologous reactions in gel precipitations against sera prepared by the same strains, were taken as an indication that antigenic substances of type specific nature were not present

The strain h 186 represented, however, an exception in that it yielded a very strong homologous precipitation This fact suggested the possible existence of a type specific antigen

Further examination of the strains by a new set of type sera resulted in the somewhat unexpected finding of four type e strains in addition to those already found, *viz* the three strains already related to type e by capsular swelling tests (Table 2), and finally strain h 186 The relation of h 186 to type e was confirmed by further experiments using sera prepared by immunization with the strains under examination against type reference antigens

According to these findings there exists two variants within type e, one variant represented by strain e 51, another by strain h 186

The fact that 1 5 volume of ethanol is sufficient for the precipitation of the type specific factors in strain h 186, while 3 volumes are needed in strain e 51 (Fig 2), agrees with what has already been stated in previous reports (*Omland* 1963 d), in which the singular position of strain e 51 in this respect is pointed out As the so called factor I is supposed to represent the main difference in the type specific make-up of the two variants, it might be speculated whether this factor is also responsible for the higher precipitability in h 186 *e g* through a co precipitation mechanism Further experiments would be needed to elucidate this It is however considered outside the scope of this study

It seems natural to correlate these results with findings reported by *Williamson & Zinnemann* (1954) pointing to the presence of a twin component type specific substance in type e Attention is also drawn to the findings described by *Rosenberg Leidy, Jaffe & Zamenhof* (1961) to the effect that the type e substance contains two monosaccharide components

Except for the fact that all the type e strains belong to the mucoid category, there has been found no correlation between the findings reported (see Fig 1) and the mucoid smooth differentiation (Table 2) It must however be mentioned that the strain h 169 showing particularly strong heterologous reactions, has been isolated from cerebrospinal fluid in a case of meningitis The strain was the only untypable meningitic strain in the original material all the remaining ones being type b (*Omland* 1963 d)

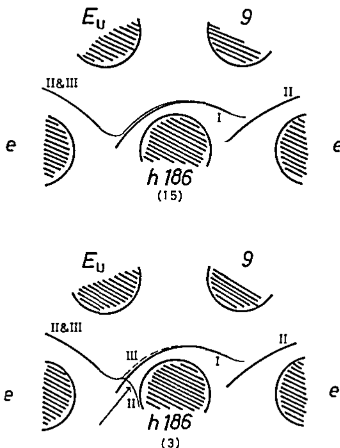


Fig 2

Differences in type specific precipitation between two type e strains

e strain e 51 type specific reference antigen

h 186 strain h 186 type specific antigen precipitated by 1 5 vol ethanol (1 5) and between 1 5 and 3 vol (3)

E_u type e reference serum kindly provided by dr Grace Leidy, New York

9 own type e serum

ly I, but also considerable amounts of II, whereas e 51 possesses mainly II (traces of I) Factor III is present in both strains, but apparently in small amounts Two fractions of the h 186 antigen have been tested, one precipitated by 1 5 volumes of ethanol (marked 1 5), and the other prepared by further precipitation in the supernatant up to 3 volumes (marked 3) Fig 2 shows that essentially all the factor II present in h 186 has been obtained in the 1 5 volume fraction (no identity reaction as concerns factor II between the two strains in lower figure, see arrow)

These findings of a complex type specific antigen in type e have been confirmed in experiments using ultrasonically treated suspensions of organisms as antigens (see a subsequent article, *Omland* 1964 a)

Acta path et microbiol scandinav 62 79-88, 1964.

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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

7 *Examination by Means of Gel Precipitation of Somatic Antigens Prepared by Various Disintegration Procedures in an M- and an R-Strain of* H influenzae

By

TOV OMLAND

Received 20 1 64

In the preceding studies (*Omland* 1963 a, b, c, d, e, 1964 a, b) the interest has been focussed on type-specific antigens and their distribution in a major material of strains

In the following some investigations shall be reported concerning mainly the non-type-specific antigens (here called *somatic* antigens)

MATERIAL

of

M

... examinations serum no 5 (anti b 51 serum) was employed

METHODS

Three disintegration ...
ment with sodium t...
Ultrasonic treatm...
horse blood agar w...
atmosphere and har...
volume of saline ...

An MSE ultrasonic apparatus was used Volumes of about 2 ml were treated each time cooling the samples in ice water during treatment ...

Treatment with sodium taurocholate (*Pittman* 1929) ...

... The mixture was incubated

SUMMARY

A selection of 17 *H. influenzae* strains, non-typable by previous gel precipitation tests, but growing in especially shiny colonies, has been examined for the eventual occurrence of capsular antigens different from the known type specific antigens. Antisera have been prepared to each strain, and the examinations have been performed by testing strains against sera in the various homologous and heterologous combinations. No substances of the nature mentioned have been found. However, complexities have been demonstrated in type e, and four of the strains have been shown to belong to a type e variant which was undetectable by the type e reference system used earlier.

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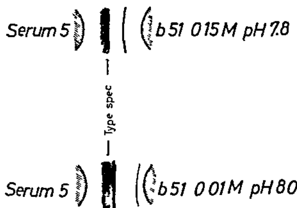


Fig 1

Gel precipitation after ultrasonic treatment for 3 minutes at pH 7 8–8 0
Effect of variation in salt concentration
Type specific precipitate partly covered by other lines (cfr later experiments)

a definite difference between physiological and hypotonic salt concentration The best distinction of lines was obtained at physiological salt concentration (Fig 1)

A new series of experiments was carried out to compare freshly treated suspensions with suspensions treated and then stored at +4° C for 10 days The centrifugation was performed at the end of the storage period In this series the following conditions were adhered to ultrasonic treatment for 3 minutes at a salt concentration of 0 15 M and a

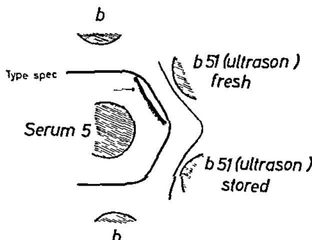


Fig 2

Gel precipitation after ultrasonic treatment for 3 minutes (0 15 M pH 7 8)
Effect of storage of antigen.
b type b reference antigen (*Omland* 1963 d) Arrow indicates precipitation due to impurities from medium (horse serum antigen)

for 1 hour in a water bath at 37° C and thereafter set up in gel precipitation experiments after high speed centrifugation as described above

Treatment with sodium carbonate The stock suspension was prepared in the same manner as described above and a 10 per cent dilution of this suspension was prepared in 1 per cent sodium carbonate (*Tunevall* 1953) After 1 hour on the desk the pH was lowered to 8 (from about 10) by means of 1 M acetic acid The same centrifugation procedure was employed as described above

Gel precipitation The technique described earlier (*Omland* 1963 c) was used

Enzyme treatment
experiments to the
1 250) in a concent
in a concentration of
of 0 1 mg per ml As seen from the used concentrations a definite excess of enzyme was aimed at in order to secure the effect The treatment was in all cases performed at 37° C for 1 hour at the standard pH described elsewhere under the respective procedures It was thus not possible to adhere to the optimal conditions pertaining to papain (tp 52° C and pH 6–6 5)

Microscopical examination The suspensions were examined microscopically before and after the various disintegration procedures in order to control the effect of the treatment Optical microscope (Gram stained smears), and in some cases also electron microscope (palladium shadowed preparations) were employed

RESULTS

a) Experiments Employing Ultrasonic Treatment

Effect of time of treatment, salt concentration, pH, and storage of antigen suspensions after treatment (strain b 51) Preliminary experiments had suggested as optimal a treatment period of 3 minutes as concerns the occurrence of precipitation lines In the following experiments parallels were set up employing respectively 2, 3, and 4 minutes of treatment The suspensions were prepared in the manner already described, and the combinations of salt concentration and pH shown in Table 1 were used

TABLE 1

Combinations of Salt Concentration and pH in Experiments on Ultrasonic Treatment

	Total salt concentration	
	0 15 M	0 01 M
pH	5 6	5 8
	5 9	6 1
	6 4	6 5
	6 9	7 1
	7 4	7 6
	7 8	8 0

Phosphate buffer was employed In the series shown in the left column (0 15 M) the proportions were 0 01 M buffer + 0 14 M NaCl In the series shown in the right column (0 01 M) the proportions were *0 005 M buffer + 0 005 M NaCl* Untreated suspensions were used as controls The samples were set up in gel precipitations against serum no 5 (homologous) and read after 2 days of immunodiffusion It was found that variations of treatment within the time interval 2 4 minutes produced no appreciable changes in the results The precipitation lines seemed also scarcely *influenced by changes in pH There was however*

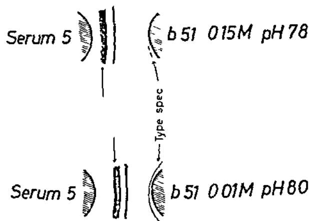


Fig 4
Gel precipitation after taurocholate treatment at pH 7 8–8 0
Effect of variation in salt concentration
Arrows indicate precipitations due partly to impurities from medium (horse serum antigens) (cfr other experiments)

In the case of bile salt treatment the trends were clear-cut, the optimal conditions being hypotonic salt concentration and high pH (0 01 M, pH 8 0) An osmotic effect is thus added to the bile salt effect A system is seen consisting of at least two lines in the middle area The line close to the antigen reservoir is due to type specific antigen

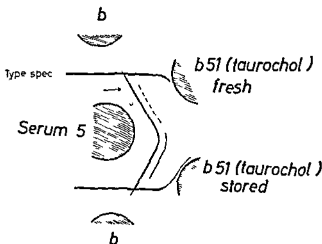


Fig 5

Arrow

pH of 7 8 This pH was chosen also with a view towards later experiments using immuno-electrophoresis, which requires a high pH The results are illustrated in Fig 2

The main features of the two patterns were equal The following differences may, however, be noted the line closest to the antigen reservoirs was distinctly stronger corresponding to the stored antigen A diffuse line close to the serum reservoir was on the other hand present only in the fresh antigen This line was shown to be caused by horse serum antigen (from the medium), by direct comparison in gel precipitation control experiments *Figs 1 and 2 show poorer resolution of* line complex near serum reservoir than indicated in later experiments

Patterns of somatic antigen lines in strain b 51 (M) and strain h 110 (R) Based on the series of experiments described above, the following conditions for ultrasonic treatment were chosen 3 minutes' treatment at 0 15 M salt concentration and pH 7 8 Experiments were carried out *in parallel using antigens prepared from each strain* The results are presented in Fig 3

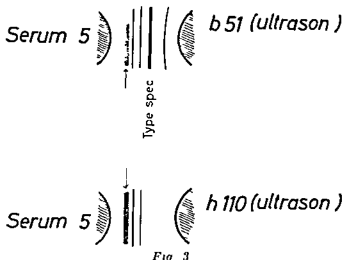


Fig 3

Gel precipitation after ultrasonic treatment (standard conditions see text)
Comparison between M strain (b 51) and R strain (h 110)
Arrows indicate precipitations due to impurities from medium (horse serum antig)

The main elements in the two patterns were similar with exception of the type specific line The line nearest to the antigen reservoir (mentioned above) could, however, not be detected in h 110

The diffuse line caused by horse serum antigen was present in both strains

b) *Experiments Employing Treatment with Sodium Taurocholate*

Effect of salt concentration, pH, and storage of antigen suspensions after treatment (strain b 51) The suspensions were prepared as already described, and the combinations listed in Table 1 were used Fig 4 illustrates the results

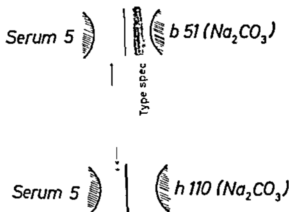
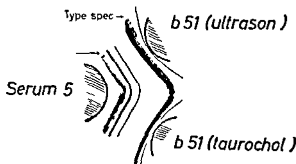


Fig 7

Cel precipitation after sodium carbonate treatment
Comparison between M strain (b 51) and R strain (h 110)
Arrows indicate prec pitations due to impurities from medium (horse serum antig)



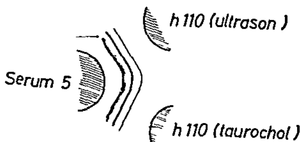


Fig 8

Cel precipitation of M and R strain
Comparison between ultrasonic treatment and taurocholate treatment of antigens
Arrows indicate precipitations due to impurities from medium (horse serum antig)

In analogy with the storage experiments described under ultrasonic treatment some examinations were performed using freshly prepared suspensions in parallel with suspensions treated with sodium taurocholate and thereafter stored at +4° C for 10 days Fig 5 shows the results

As in the ultrasonic experiments a diffuse line closest to the serum reservoir disappeared after storage This line was shown to be due to horse serum antigen (cfr above)

Patterns of somatic antigen lines in strain b 51 (M) and strain h 110 (R) The optimal conditions for bile salt treatment (0 01 M pH 8 0) were used in these examinations which were carried out in parallel in the two strains The results are seen from Fig 6

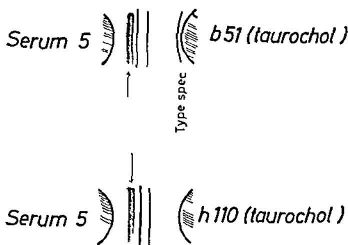


Fig 6

Gel precipitation after taurocholate treatment (standard conditions see text) Comparison between M strain (b 51) and R strain (h 110) Arrows indicate precipitations due to impurities from medium (horse serum antig)

As in the analogous ultrasonic experiments the main elements in the two precipitation patterns are the same excepting the type specific line which is weak even in strain b 51

c) *Experiments Employing Treatment with Sodium Carbonate*

Patterns of somatic antigen lines in strain b 51 (M) and strain h 110 (R) The treatment was carried out as described under Methods The results are seen in Fig 7

A similar pattern to the one already recorded by the two previous disintegration methods could be seen in the middle area between the reservoirs Also in these experiments no line close to the antigen reservoir could be detected

treatment than after taurocholate treatment, suggesting that the former treatment yielded a more highly diffusible antigen than the latter, which might also scarcely be expected to have any direct effect on the type specific polysaccharide (cfr immuno-electrophoretic examinations, *Omland* 1964 b)

A diffuse, weak precipitation near the serum reservoir proved to be due to horse serum antigen This was also in accordance with the results of enzyme treatment (see below) This precipitation tended to disappear gradually on storage of the antigen mixture (Fig 2, Fig 5) It may be be assumed that autolytical processes were active during storage (cfr strengthening of the line closest to the antigen reservoir in ultrasonically treated antigens)

Treatment of the final antigen preparations by proteolytic enzymes produced no demonstrable effect on the bacterial antigen lines DNAase was also without detectable influence On the other hand the proteolytic enzymes affected strongly the horse serum antigen precipitation as already mentioned

Several earlier works on non type specific *H influenzae* antigens have been reported (see *Omland* 1963 a) Of the more recent studies the investigation made by *Tunevall* (1953) on sodium carbonate extracted antigens must be mentioned At least two non-type specific *H influenzae* antigens have been reported earlier (the so called M and P substances, *Boyd* 1956 p 197) *Dubos* (1942) described the finding of an antigen supposed to be of endotoxin nature

After the above examinations it is unlikely that the demonstrated antigens are of protein nature like the M and P substances It seems more probable that the antigens are at least partly of polysaccharide nature

Keeping Dubos finding in mind, a relation to the endotoxins might be suspected Further studies performed by aid of immuno-electrophoresis are to be published shortly

SUMMARY

An M strain (type b) and an R-strain of *H influenzae* have been examined in gel precipitation experiments against an anti b serum after disintegration by ultrasonic oscillations, by bile salt, and after treatment by [illegible]
(in the M-[illegible]
Because th[illegible]
by proteol[illegible]
of protein [illegible] such as the M and P substances reported by earlier authors It is thought that they may at least in part be of polysaccharide nature (related to endotoxins?)

Comparison of the Somatic Antigen Line Patterns as Revealed by the three Methods

Direct comparison has been made between the patterns produced by ultrasonic and taurocholate treatment in gel precipitation experiments illustrated in Fig 8 The narrow line closest to the antigen reservoir, previously seen *only after ultrasonic treatment*, has been *demonstrated* for the first time also after taurocholate treatment

Based on these experiments a general pattern of somatic antigen lines may be postulated, and is hereby presented schematically (Fig 9).

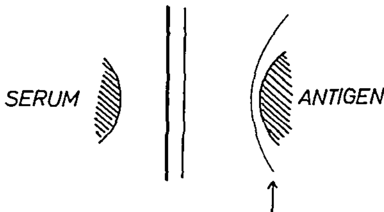


Fig 9

Schematic pattern of non-type specific (somatic) lines as revealed by gel precipitation experiments in two H influenzae strains (M and R) Arrow indicates precipitation produced only by M strain

Enzyme treatment The treatment by trypsin, papain, and DNAase proved without demonstrable effect on the different line systems with one exception, *viz* the described horse serum line nearest to the serum reservoir This line was weakened or completely removed in experiments using trypsin and papain

DISCUSSION

Examinations have been performed with the purpose of studying the non-type-specific antigens in a mucoid and a rough strain of *H influenzae* Three different disintegration methods have been employed ultrasonic treatment, treatment with sodium taurocholate, and treatment with sodium carbonate In addition, the effect of storage of antigen has been examined

Ultrasonic and taurocholate treatment yielded essentially similar results, being directly comparable in gel precipitation experiments (Fig 8) The pattern caused by somatic antigens consisted thus mainly of 2 sharp lines— in the mucoid strain 3 lines—while the precipitation after sodium carbonate treatment consisted of only one line The type specific line present in preparations from the mucoid strain was stronger and situated farther from the antigen reservoir after ultrasonic

treatment than after taurocholate treatment, suggesting that the former treatment yielded a more highly diffusible antigen than the latter, which might also scarcely be expected to have any direct effect on the type specific polysaccharide (cfr. immuno-electrophoretic examinations, *Omland* 1964 b).

A diffuse, weak precipitation near the serum reservoir proved to be due to horse serum antigen. This was also in accordance with the results of enzyme treatment (see below). This precipitation tended to disappear gradually on storage of the antigen mixture (Fig. 2, Fig. 5). It may be be assumed that autolytical processes were active during storage (cfr. strengthening of the line closest to the antigen reservoir in ultrasonically treated antigens).

Treatment of the final antigen preparations by proteolytic enzymes produced no demonstrable effect on the bacterial antigen lines. DNAase was also without detectable influence. On the other hand the proteolytic enzymes affected strongly the horse serum antigen precipitation as already mentioned.

Several earlier works on non type specific *H. influenzae* antigens have been reported (see *Omland* 1963 a). Of the more recent studies the investigation made by *Tunevall* (1953) on sodium carbonate extracted antigens must be mentioned. At least two non-type specific *H. influenzae* antigens have been reported earlier (the so called M and P substances, *Boyd* 1956 p. 197). *Dubos* (1942) described the finding of an antigen supposed to be of endotoxin nature.

After the above examinations it is unlikely that the demonstrated antigens are of protein nature like the M and P substances. It seems more probable that the antigens are at least partly of polysaccharide nature. Keeping Dubos' finding in mind, a relation to the endotoxins might be suspected. Further studies performed by aid of immuno electrophoresis are to be published shortly.

SUMMARY

An M strain (type b) and an R-strain of *H. influenzae* have been examined in gel precipitation experiments against an anti-b serum after disintegration by ultrasonic oscillations, by bile salt, and after treatment by 1 per cent sodium carbonate. A system of 2 precipitation lines (in the M strain 3 lines) has been established as being of somatic origin. Because the lines remained unaffected despite treatment of the antigens by proteolytic enzymes (and DNAase), it appears unlikely that they are of protein nature such as the M and P substances reported by earlier authors. It is thought that they may at least in part be of polysaccharide nature (related to endotoxins?).

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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

8 *Examinations of Ultrasonically Prepared Haemophilus Antigens by Means of Immuno Electrophoresis*

By

TOV OMLAND

Received 20 1 64

In the present, final part of these serological studies on Haemophilus immuno electrophoretic technique has been employed In addition to *H influenzae*, *H haemolyticus*, *H parainfluenzae*, and *H parahaemo-* [illegible] limited number of strains of

[illegible] estigations the reader is referred to the first report of the series (*Omland* 1963 a) The purpose of the immuno electrophoretic examinations was besides to inquire further into the serology of the type specific antigens of *H influenzae*, to study the non-type specific antigens of that species, and finally to search for related antigens in the mentioned other species of *Haemophilus*

It is considered appropriate to recapitulate some of the earlier studies on non type specific H influenzae antigens Most of these studies serve to illustrate a confusing heterogeneity Thus *Park*, *Williams* & *Cooper* (1918, cit *Wilson* & *Miles* 1955, p 909) found only 4 identical pairs among 160 strains *Yabe* (1921) and *Krah* (1930) similarly confirmed the diversity among *H influenzae* S-strains *Yagi* (= *Iizuka*) (1935, 1938) had to establish 46 antigenic groups in a material of about two hundred strains *Platt* (1937), in a material of 87 strains, likewise failed to attain an adequate serological classification Two non-type-specific antigens of protein nature, the so-called P and M substances have been reported (*Boyd* 1956, p 197) *Dubos* (1942) described an antigenic substance supposedly of endotoxin character in *H influenzae*

MATERIAL

Strains The strains examined have been selected from the original material (*Omland* 1963 a) Both typable and untypable strains have been included A review f the strains examined is presented in Table 1

Sera The sera have been selected from a major collection because of satisfactory properties in gel precipitation and immuno electrophoresis experiments The sera are listed in Table 2

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Oslo Norway (Head Professor S D Henriksen M D)

SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

8 *Examinations of Ultrasonically Prepared Haemophilus Antigens by Means of Immuno Electrophoresis*

By

TOV OMLAND

Rece ved 20 1 64

In the present final part of these serological studies on Haemophilus immuno electrophoretic technique has been employed In addition to *H influen ae H haemolyticus H parainfluen ae* and *H parahaemo lyticus* have been studied although only a limited number of strains of the three latter species has been available

Concerning the background of these investigations the reader is re ferred to the first report of the series (*Omland* 1963 a) The purpose of the immuno electrophoretic examinations was besides to inquire further into the serology of the type specific antigens of *H influen ae* to study the non type specific antigens of that species and finally to search for related antigens in the mentioned other species of *Haemophilus*

It is considered appropriate to recapitulate some of the earlier studies on non type specific H influenzae antigens Most of these studies serve to illustrate a confusing heterogeneity Thus *Parl Williams & Cooper* (1918 cit *Wilson & Miles* 1955 p 909) found only 4 identical pairs among 160 strains *Yabe* (1921) and *Krah* (1930) similarly confirmed the diversity among *H influen ae* S strains *Yagi* (— *It uka*) (1935 1938) had to establish 46 antigenic groups in a material of about two hundred strains *Platt* (1937) in a material of 87 strains likewise failed to attain an adequate serological classification Two non type specific antigens of protein nature the so called P and M substances have been reported (*Boyd* 1956 p 197) *Dubos* (1942) described an antigenic substance supposedly of endotoxin character in *H influen ae*

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Sera The sera have been selected from a major collect on because of sat sfactory propert es in gel prec p tation and immuno electrophores s experiments The sera are l sted in Table 2

TABLE 1

Strains Examined by Immuno Electrophoresis

		Number of strains
H influenzae type strains type	a	2
(incl reference strains)	, b	33
	, c	1
	d	3
	, e	6
	, f	7
H influenzae non typable strains		21
H haemolyticus		1
H *parainfluenzae*		1
H parahaemolyticus		16
	Total	91

TABLE 2

Sera Employed in Immuno Electrophoretic Examinations

		Number of sera	Designations of sera						
Anti H influenzae type specific sera	a	1	3						
	b	1	5						
	c	1	15						
	d	1	16						
	e	5	9	*19*	*23*	65	66		
	f	7	10x	*28*	21	*31*	*33*	*34*	*47*
Anti H influenza non type specific sera		6	*35*	*36*	*38*	*39*	*42*	*51*	
Total		22							

Designation with italic serum used in the examination of *all* strains listed in Table 1

METHODS

Disintegration Preliminary experiments have been performed using several dis integration procedures but the present report has been based on the use of one single procedure or ultrasonic treatment It seemed justified to assume that con ditions giving the best results in gel precipitation experiments would also be applicable in immuno electrophoretic studies (*Omland* 1964 b)

The organisms were seeded from freezedried state and grown for 18 20 hours on chocolate agar plates (heated horse blood agar without the addition of ascitic fluid) in a humid atmosphere and thereafter harvested into a minimum of fluid (physio logical saline) A 20 per cent dilution of this suspension was prepared in buffered saline (0 14 M NaCl + 0 01 M phosphate buffer) at pH 8 0 The optical transmission of the suspension was registered in each experiment by aid of a Beckman mod C colorimeter using a green filter (524 mμ) The measurements were performed after a fourfold dilution of the said *20 per cent suspension* (*i e in 5 per cent of the ori* ginal suspension) in order to obtain a convenient level of measurement values After ultrasonic treatment the sample—and the untreated control—was centrifuged at high

TABLE 3

Properties of Immuno Electrophoretic Precipitation under Different Conditions

Buffer	Total salt concentration	pH 7.0	7.5	8.0	8.6
Veronal (EKB)	0.15 M	—	—	—	Fairly distinct Fast migration
Veronal HCl	0.05 M	—	—	Fairly distinct Fast migr	—
Phosphate (+NaCl)	0.05 M		—	*Fairly distinct Slow migr	—
Phosphate	0.05 M	-	*Distinct Slow migr	*Distinct Slow migr	—
Phosphate (+NaCl)	0.01 M	Distinct Slow migr	Distinct Moderate migr	Distinct Fast migr	—
Phosphate	0.01 M	—	Distinct Moderate migr	Distinct Fast migr	—

* Strong generation of heat — not examined

speed (10 000–12 000 g) for 30 minutes and the supernatant subjected to immuno electrophoretic analysis

Immuno electrophoresis An LKB immuno electrophoresis apparatus designed for microscopical slides (26 × 76 mm) has been used Based on the experimental con ditions recommended by the manufacturer a series of experiments was carried out to adapt the technique to the special task of examining *Haemophilus* antigens Except for minor changes the recommended basic conditions for electrophoresis and immunodiffusion were adhered to Thus 45 minutes' electrophoresis at 250 V, and 20 hours' immunodiffusion at room temperature were employed One per cent Difco Special Agar Noble was used for preparing the gel, which also contained Merthiolate Lilly (1 10 000) and methylorange (3 100 000) The experiments were designed to select the optimal pH and buffer salt mixture A homologous system consisting of strain b 51 against serum no 5 was used as a representative test system The recording of results was carried out by photographing the precipitation lines according to principles described earlier (*Omland* 1963 b) No staining was employed The results are summarized in Table 3

Compared to the conditions recommended by the manufacturer as good or even better distinction and separation of lines have been obtained at low salt concen trations It was beforehand considered desirable to ensure conditions as near as possible to the conditions under which most of the previous gel precipitation studies had been carried out, so that the results might, as far as possible be comparable The following composition of the buffer salt mixture was then chosen 0 01 M (0 005 M NaCl + 0 005 M phosphate buffer) and pH 8 0 The ampereage remained fairly constant during each experiment using this buffer salt mixture It varied also only slightly from one experiment to another being in all instances within the limits 18 and 22 mA

It was found in separate experiments that several sera contained antibodies against horse serum antigens (cfr also *Omland* 1964 b) The corresponding undesired precipitations could however, be completely prevented by adding horse serum to the gel This was done in all subsequent experiments (in a concentration of 1 per cent of the gel) At first each experiment was performed in parallel in gel with and without horse serum Later the experiments in gel without horse serum were omitted as they furnished no extra information The addition of human ascitic fluid was unnecessary as this was not employed as a constituent in the chocolate agar in the present series of examinations (cfr *Omland* 1963 c)

Enzyme treatment In a set of experiments the supernatants from ultrasonic treat ment were exposed to the action of trypsin (Trypsin Difco 1 250) in a concen tration of 0 05 0 1 mg per ml at 37° C for 1 hour before immuno electrophoretical analysis

Microscopical examination of the suspensions was performed before and after ultrasonic treatment

RESULTS

a) *Type Specific H influenzae Antigens*

In all instances the type specific antigens were found not only in the ultrasonically treated suspensions, but also in the controls, *i e* in supernatants obtained after centrifugation of untreated bacterial sus- pensions In this respect they differed clearly from non-type-specific antigens, which, with one exception (cfr below), were detectable in solution only after ultrasonic treatment

As a check of the type specific properties experiments were performed cross matching all available type reference strains against the respec- tive type sera In each of these experiments solutions of type specific reference antigens were used as additional controls These type re- ference antigens were the same as those employed earlier (*Omland* 1963 c)

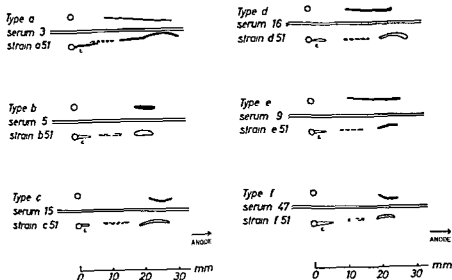


Fig 1

Immuno-electrophoretic patterns of type specific *H influenzae* antigens (schematic)
Experiments using ultrasonically treated antigens and controls are superposed for comparison
Above serum groove type reference antigen (*Omland* 1963 c)
Below serum groove antigen prepared by ultrasonic treatment (continuous outline) antigen prepared by simple dissolution (dotted outline)
Non type specific ε antigen also shown

The three modifications of type specific antigens thus at disposal for examination behaved, as might be expected, in sligthly different ways 1) Antigens obtained by simple dissolution (controls) These antigens demonstrated the greatest homogeneity, perhaps with the exception of the type a antigen, which precipitated in an extended line, suggesting a great variation range in electrophoretic mobility 2) Antigens obtained by ultrasonic treatment In these antigens there were, more or less in all types, signs of inhomogeneity in the form of "tail" formation towards the antigen reservoir, apparently forming a connection with the so-called ε line (see below) The type a antigen showed the same tendency as mentioned above, precipitating in a line of considerable length 3) Reference antigens obtained by ethanol precipitation and phenol treatment (*MacPherson* 1948, *Omland* 1963 c) These antigens presented two different patterns, one characterized by drawn-out precipitation lines (types a, d, and e), the other characterized by precipitation spots (types b, c, and f)

The general acidic properties of the type specific antigens (*Zamenhof, Leidy Fitzgerald, Alexander & Chargaff* 1953, *Zamenhof & Leidy* 1954, *Rosenberg, Leidy, Jaffe & Zamenhof* 1961) were clearly demonstrated as they migrated rapidly towards the anode

Representative experiments in this series are shown in Fig 1

In view of the particular characteristics revealed by the types e and f (*Omland* 1964 a) it was decided that immuno-electrophoretic examinations should be carried out in all available type e and type f strains against the respective type sera The results of these examinations are reviewed in Table 4

TABLE 4
Type Specific Precipitation in Immuno Electrophoresis Type e and f

Type e strains	Type e antisera				
	9	19	23	65	66
e 51	+*	+*	+*	trace	trace
e Mo	trace	trace	trace	+	+
h 48	trace	trace	trace	+	+
h 131	trace	trace	trace	+	+
h 145	trace	trace	trace	+	+
h 186	trace	trace	trace	+*	+*

Type f strains	Type f antisera						
	10x	18	21	31	33	34	47
f 51	+*	trace*	+*	trace*	trace*	+*	+
f Di	trace	trace	+	trace	trace	+	+*
h 43	+	trace	+	trace	trace	+	+
h 128	+	trace	+	trace	trace	+	+
h 147	+	trace	+	trace	trace	+	+
h 178	+	trace	+	trace	trace	+	+

* indicates reaction between serum and strain used for immunization

It is seen that the type e strains are divided into two varieties, one represented by the type reference strain e 51, yielding strong type specific precipitations against sera no 9, 19, and 23, but not against sera no 65 and 66, the other represented by the rest of the examined strains, yielding strong type specific precipitations against sera no 65 and 66, but not against sera no 9, 19, and 23

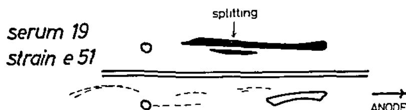


Fig 2
Immuno electrophoretic pattern showing splitting of type specific precipitation type e
Above serum groove type e reference antigen (*Omland* 1963 c)
Below serum groove antigen prepared by ultrasonic treatment Continuous outline type specific precipitation Dotted lines non type specific precipitation

As indicated in gel precipitation experiments (*Omland* 1964 a), type e possesses a multiple component type specific antigen This feature is also reflected in immuno electrophoresis showing a splitting of the type specific precipitate (Fig 2)

Within type f it has not been possible to demonstrate more than one serological variety (cfr however the f Di serum no 10x reaction Table 4) Within this type there are however also indications of a complex type specific antigen (*Omland* 1963 c Fig 6) a finding which was confirmed in immuno electrophoresis The system strain f Di against serum no 21 is illustrative in this respect (Fig 3)

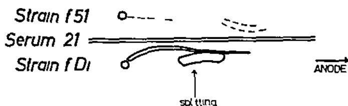


Fig 3

Immuno electrophoretic pattern showing splitting of type specific precipitation type f
Above serum groove type f reference antigen (*Omland* 1963 c) (homologous system)
Below serum groove antigen prepared from another f strain (f Di) by simple dissolution (heterologous system)

b) *Non Type Specific H influenzae Antigens*

Seventy three strains have been examined by aid of the 10 selected sera

In order to create a basis for the final analysis of the antigenic pattern of each strain or group of strains the different precipitation patterns produced by the single sera were examined in an attempt to establish the characteristics of each serum This was done by measuring the approximate distance of each line (or line complex) from the antigen reservoir With few exceptions the lines were arc shaped and the distances could be measured from the point on the arc nearest to the serum groove and parallel to this Minor differences in electrical strength of field and in the scale of optical recording had to be taken into consideration Equally important was however the peculiarity of outline pertaining to each precipitation line (or line complex) These purely qualitative properties were in most instances so characteristic that it was easy to establish a precipitation pattern for the single sera representing the "total make up of precipitins active in each

From the patterns of the different sera it was possible to fit together a hypothetical total pattern to be used as an aid in the final analysis of strains The line patterns belonging to each serum and the hypothetical total pattern are seen in Fig 4

It is seen that all sera yielded a strong complex of lines on the

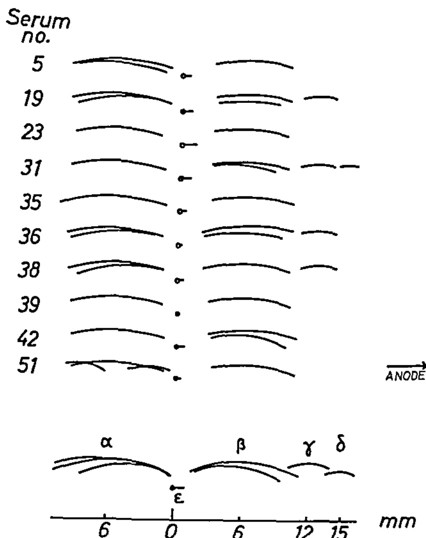


Fig 4

Schematic immuno electrophoretic patterns of non type-specific (somatic) precipitations characteristic of various sera Synthesis into theoretical total pattern

cathode side of the antigen reservoir, provisionally called the α complex The complexes situated on the anode side showed greater variations from one serum to another Thus serum no 31 presented a multitude of line complexes, provisionally called the β, γ, and δ complexes, in addition to the ε line, originating in the antigen reservoir itself Serum no 35 is an example of a serum yielding few precipitation lines, mainly the α and β line complexes

The analysis of the non type-specific antigens in each strain was performed in the same way as described above concerning the fitting-

together of the hypothetical total precipitation pattern By superposing the patterns produced in a single strain by the different sera, a picture of the antigenic equipment of that strain was brought forward

The presentation of this rather extensive collection of single results must necessarily be problematic In the following illustration (Fig 5) the trends revealed by the present investigations have been placed in relation to the grouping used above (Table 1) The results have been expressed mainly in two ways, firstly in the form of numbers of antigens (as demonstrated by numbers of lines) within the different groups of strains, secondly in the form of schematic line patterns representative of each group

In type a a comparatively simple pattern of lines was found A most interesting feature was the demonstration of a highly soluble, non-type-specific antigenic component (precipitation indicated by arrow), possessing an electrophoretic mobility similar to the δ complex, but otherwise different from this complex It was thus only produced by one of the listed sera, serum no 5 which was practically devoid of precipitin corresponding to the δ-complex (Fig 4) The solubility, a so far unique property among non type-specific antigens, was demonstrated in the untreated controls, which yielded a similar line as sole precipitation, however, located somewhat nearer to the antigen reservoir

In type b the pattern included mostly a double-line α complex, a double line β complex, and a single-line γ precipitation In 3 strains a δ line was clearly present The suspensions of these 3 strains were of medium (not maximal) optical density

In the single type c strain available a pattern similar to the type b pattern was found, except for the α complex, which consisted af a single line

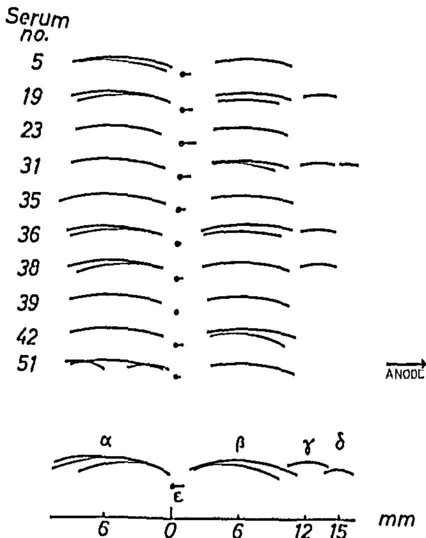
The type d pattern was similar to the type c pattern

Type e was particularly well equipped showing double-line complexes both in the *α, β and γ location, and in addition* a clear δ line in one strain

In type f a difference was noted between strain f 51 and the other strains While the other f strains presented a complete pattern of lines similar to the general type e pattern, the f 51 strain apparently possessed fewer antigens mainly located in the α and β complexes Because of poor growth this strain gave, however, a thin suspension, so that the result may at least in part be explained technically

The group of non-typable strains revealed a pattern which did not differ greatly from the general pattern shown by type strains Mention should nevertheless, be made of the remarkable complexity of the α complex The β and γ complexes were generally more poorly developed, while the δ complex was not present (or only as trace in 2 strains) The ε line could be clearly made out in all the above mentioned strains

The only R strain (*Omland 1963 a*) was also examined All the line complexes were distinct except the ε line, which could be traced in



Synthesis of total pattern (schematic).

Fig. 4

Schematic immuno-electrophoretic patterns of non-type-specific (somatic) precipitations characteristic of various sera. Synthesis into theoretical total pattern.

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the precipitation by one serum only All complexes were represented by single lines, with the exception of the α complex, which had two lines

A certain variation from strain to strain within each group was registered, as can be read out of Fig 5 (variation limits of numbers of lines) and Table 5 While the α and β complexes were constantly present in all strains, inconstancy was noted in the γ and δ complexes The ε line was present in all strains, although only as a trace of precipitation in the R-strain

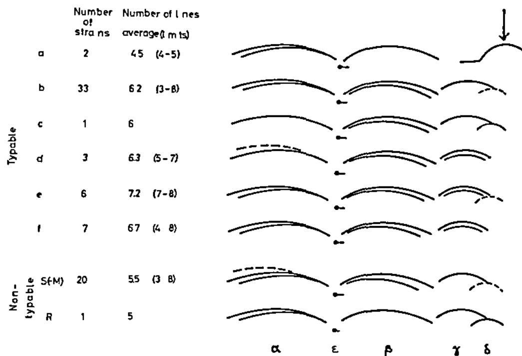


Fig 5

Schematic immuno electrophoretic patterns of non type specific precipitations representative of various groups of *H influenzae* strains
Non type specific soluble antigen indicated by arrow (see text)

TABLE 5

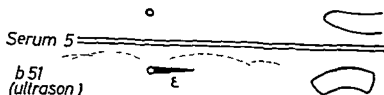
Subgrouping of H influenzae *Strains according to Non Type Specific Pattern in Immuno Electrophoresis*

Type	Number of strains	Number of different patterns
a	2	2
b	33	11
c	1	
d	3	3
e	6	3
f	7	6
Untypable	20	8

TABLE 6

Optical Transmission Values of Suspensions of Organisms before Ultrasonic Treatment (Beckman Colorimeter Mod C)

H influenzae type	Number of strains	Transmission values (%)	
		average	limits
H influenzae type a	2	36	33 39
b	33	27	18–36
c	1	34	
d	3	30	26–34
e	6	27	24–32
f	7	29	21 39
H influenzae untypable	20	32	20–45
H haemolyticus	1	49	
H parainfluenzae	1	86	
H parahaemolyticus	16	46	33 70



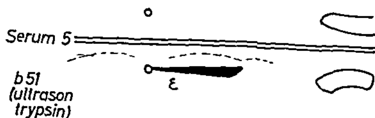


Fig 6

the precipitation by one serum only. All complexes were represented by single lines, with the exception of the α complex, which had two lines.

A certain variation from strain to strain within each group was registered, as can be read out of Fig. 5 (variation limits of numbers of lines) and Table 5. While the α and β complexes were constantly present in all strains, inconstancy was noted in the γ and δ complexes. The ε line was present in all strains, although only as a trace of precipitation in the R-strain

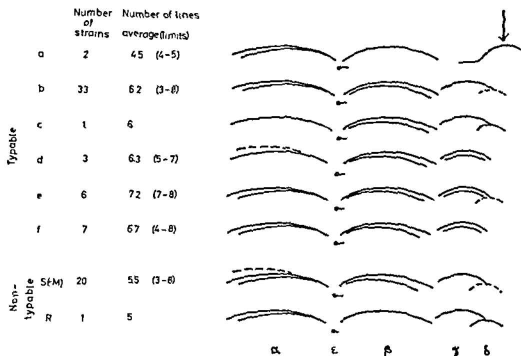


Fig 5

Schematic immuno-electrophoretic patterns of non type-specific precipitations representative of various groups of *H influenzae* strains
Non-type specific soluble antigen indicated by arrow (see text)

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Subgrouping of H influenzae *Strains according to Non Type-Specific Pattern in Immuno Electrophoresis*

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H influenzae, untypable	20	32	20–45
H haemolyticus	1	49	
H parainfluenzae	1	86	
H parahaemolyticus	16	46	33–70

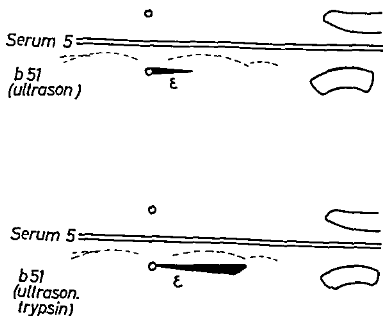


Fig 6

Effect of trypsin treatment of ultrasonically prepared antigen in a homologous

Cor

n groove, type b

structure?) as indicated by the location of the precipitates near the electrophoretic axis

The complexity of the type e antigen demonstrated elsewhere, has been confirmed by immuno-electrophoretic technique By this technique it has also been possible to divide the type e strains into two variants Previous gel precipitation analysis of representatives of these two variants (*Omland* 1964 a) suggests that the variant occurring most frequently in this material (represented by strain h 186) is equipped with a more complete type specific antigen (considerable amounts of two factors) than the other variant (strain e 51, mainly one factor) The fact that the strains belonging to the most common type e variant behaved differently when tested in gel precipitation against the original type reference system, strain e 51/serum no 9 (two strains typable, three strains untypable (*Omland* 1963 c), suggest, however, quantitative differences even within that variant These findings are, on the whole, in good agreement with the findings reported by *Williamson & Zinnemann* (1954) *indicating that the complete type e antigen consists* of two factors, e 1 and e 2, while there occurs also strains possessing only e 2

In type f the findings also *indicate a complex type specific antigen* Thus complexities are apparent in phenol treated antigen (MacPherson's method (*Omland* 1963 c)) as well as in untreated antigen solutions (Fig 3)

It is, however, in the field of non-type-specific H influenzae antigens that the most extensive results have been secured in the present study They lead to the establishment of a basic system of lines or line complexes, provisionally designed α, β, γ, δ, and ε With the exception of the γ and δ complexes, the different components appear to be present with remarkable regularity in the strains examined This pertains to type strains as well as non-type-specific strains, and also to the R-strain studied

Except for the particular finding in the two type a strains (cfr below), all non-type-specific antigens detected have been found only in ultrasonically treated suspensions of organisms, and not in untreated controls This fact, and the fact that they are generally present even in the R-strain, *indicates the true somatic character of these antigens*, *i e* that they are firmly bound to, and eventually deeply situated in, the bacterial body, in contrast to the type specific antigens which are easily obtained by simple dissolution in accordance with their capsular character

An outstanding feature in the serology of non-typable *H influenzae* strains has been the repeated finding of an extreme heterogeneity (cfr references cited in the introduction) It was natural to search among the present results for an explanation of these findings The simplest explanation would be to assume that the registered strain to-strain differences, notably seen in the γ *and* δ *complexes, are responsible for*

the said heterogeneity A more theoretical explanation is to postulate the presence of variable superficial masking substances, antigenic (cfr the vi antigen in *Salmonella*) or non antigenic under the experimental conditions Naturally it is also thinkable that the differences underlying the heterogeneity reported by earlier authors are due to antigens not demonstrable by the methods used in the present work It is thus recognized that as far as antigens at or near the bacterial surface are concerned, precipitation reactions are generally less sensitive than agglutination methods, on which the earlier results are based It seems, however, improbable that the antigenic differences demonstrated previously should entirely escape registration by the methods employed in the present work

As a final compromise in this reasoning one might postulate the heterogeneity as being due to the occurrence of a highly varied antigenic make up, however further complicated by differences in superficial masking substances

The possibility of a variable masking effect by some less antigenic (or non antigenic) surface substance (or substances), gains support in the evidence of a non-type specific, soluble surface substance which has been demonstrated in the two type a strains These strains grew in extraordinarily slimy colonies The said substance was demonstrated by aid of a type b antiserum, so that the same substance, or a closely related one, must be present even in the type b strain used for immunization (b 51), however in amounts undetectable by direct precipitation As a consequence it is also necessary to assume a greater immunogenicity in the type b strain

Even if further evidence concerning a more widespread non-type specific surface substance (eventually non-antigenic) is lacking, it seems appropriate at this point to revert to one of the main questions posed in the introduction to the present series of works (*Omland* 1963 a), in which the possible existence of particular superficial antigen(s) was brought forward in order to explain the shiny colonial appearance of the S-strains

The purpose of using *H influenzae* sera in the study of other *Haemophilus* species was to search for eventual common antigens Reservations must be made in the interpretations because of difficulties in securing abundant and homogenous growth

The results show that several of the main antigen complexes found in *H influenzae* are also present in *H parahaemolyticus* In the two other species, *H haemolyticus* and *H parainfluenzae*, the sparse material allows no further conclusions In *H parahaemolyticus* it may be stated however, that the β complex, mostly in the form of a single line, is present in all strains The α complex is also present in a majority of strains, generally in the form of one line Except for these two mentioned complexes the presence of lines is very inconstant from strain to strain In two strains even the ε line is present

structure?) as indicated by the location of the precipitates near the *electrophoretic* axis

The complexity of the type e antigen demonstrated elsewhere, has been confirmed by immuno-electrophoretic technique By this technique it has also been possible to divide the type e strains into two variants Previous gel precipitation analysis of representatives of these two variants (*Omland* 1964 a) suggests that the variant occurring most frequently in this material (represented by strain h 186) is equipped with a more complete type specific antigen (considerable amounts of two factors) than the other variant (strain e 51, mainly one factor) The fact that the strains belonging to the most common type e variant behaved differently when tested in gel precipitation against the original type reference system, strain e 51/serum no 9 (two strains typable, three strains untypable (*Omland* 1963 c), suggest, however, quantitative differences even within that variant These findings are, on the whole, in good agreement with the findings reported by *Williamson & Zinnemann* (1954) indicating that the complete type e antigen consists of two factors, e 1 and e 2, while there occurs also strains possessing only e 2

In type f the findings also indicate a complex type specific antigen Thus complexities are apparent in phenol treated antigen (MacPherson's method (*Omland* 1963 c)) as well as in untreated antigen solutions (Fig 3)

It is, however, in the field of non-type-specific H influenzae antigens that the most extensive results have been secured in the present study They lead to the establishment of a basic system of lines or line complexes, provisionally designed α, β, γ, δ, and ε With the exception of the γ and δ complexes, the different components appear to be present with remarkable regularity in the strains examined This pertains to type strains as well as non-type-specific strains, and also to the R-strain studied

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The results, notably those attained in the present and final part, justify an attempt at partial answers to the two main questions posed in the first part of the series

To the first question of whether the S-form possesses superficial antigen(s) explaining better its shiny colonial morphology, the following comments may be made Evidence has been obtained of the presence in a few M strains (type a and b) of a highly soluble, superficial (?), non type-specific antigenic substance Even though this finding does not lend itself to generalization, it might nevertheless be speculated if similar substances are more widespread in *H influenzae* The failure to detect such hypothetic substances may be explained by assuming either that they occur generally in very small amounts, that they are unstable, or that they are not antigenic under the employed experimental conditions (cfr above on the suggested greater immunogenicity in the type b strain)

To the second question of whether the *H influenzae* variation pattern is reflected in changes in somatic antigens the following comments are offered There seems to be no direct correlation between somatic antigenic equipment and M S R variation The strain to-strain differences are, however, most irregular, and thus in conformity with earlier investigations

In future work a natural task would be to inquire further into the question of the postulated non type specific superficial substance(s), for instance by aid of serological adjuvant technique

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These results are remarkable in view of the contrast, especially in morphological properties, between *H. parahaemolyticus* and *H. influenzae*.

SUMMARY

Antigens prepared by ultrasonic treatment of 52 type strains and 21 non-typable strains of *H. influenzae*, and 18 strains of other *Haemophilus* species, have been studied by aid of 22 anti-H influenzae sera in immuno-electrophoretic experiments.

The properties of the *H. influenzae* type specific antigens have been investigated, and complexities demonstrated previously, notably in type e, have been confirmed.

The non-type-specific antigens have been studied as visualized by a pattern of precipitation lines which appeared basically the same in all *H. influenzae* strains, however with a tendency to irregular variations in certain minor details. The fact that antigens from strains of other *Haemophilus* species yielded precipitations with the used sera, was taken as a proof of more widespread common antigenic factors

In a few strains displaying particularly slimy colonies (type a) a non-type-specific, highly soluble antigen was detected. It is pointed out that the discovered substance may be a key to the explanation of certain morphological and other features peculiar to the *H. influenzae* S-form.

GENERAL COMMENTS

The first five parts in this series of studies have been concerned with the search for type specific antigens in the material of strains and have confirmed earlier investigations showing that only M-variants possess such antigens, while the S-form, although being the most frequently occurring variant, is devoid of them During this work particular importance has been placed in the methodological basis, because a definite negative answer to the question whether the S-variants contain type specific antigen, was decisive for the planning of further work

In the sixth part a group of untypable strains with especially shiny colonial appearance has been examined for eventual superficial antigens other than the known type specific antigens No such substance was detected by the used methods However, complexities within certain serological types, notably type e, were explored

In the seventh, and in the eighth and final part, mechanical (and other) disintegration methods have been employed in addition to the previously used chemical extraction procedures, in an effort to obtain the broadest possible display of antigens, especially of somatic nature

Thus ultrasonic disintegration in connection with immuno-electrophoresis has been used in a number of M-strains, S-strains, and an R-strain of *H influenzae* A limited number of strains of other *Haemo-*

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COMPARISON BETWEEN SOME STREPTOMYCIN-RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS PREPARED IN VITRO AND THEIR PARENT STRAINS

3 *Immunological Studies*

By

LENNART WAHLSTROM

Received 10 i 64

In previous papers of this serie (*Norkrans & Wahlstrom* 1962, *Wahlstrom & Norkrans* 1963) penicillin G resistent strains of *Staph aureus* and their parent strains were examined by means of the double diffusion agar technique The results showed that the antigenic pattern of a sensitive strain underwent changes on acquiring penicillin-G resistance *in vitro* It was also found that this was not valid for a penicillinase producer In this paper, the study has been extended to cover also streptomycin-resistant strains, as not much has been reported about serological investigations of this type of antibiotic resistance

The effect of streptomycin on bacteria has been examined by numerous workers Some effects analogous to those of penicillin seem to exist *e g* the excretion of purine nucleotides in the presence of streptomycin and the defects it causes on the formation of the membrane (*Roth et al* 1960 *Anand & Davis* 1960, *Feingold & Davis* 1962) *Hancock* (1960), however, found no evidence of leakage of these compounds, using *Staph aureus* instead of *E coli*

In their unitary hypothesis, *Spotts & Stanier* (1961) propose that sensitive strains have a high affinity for binding streptomycin on the ribosomes thus blocking the attachment of messenger RNA's On the other hand, the structure of the ribosomes of the resistant strains are different having no or only a slight affinity for streptomycin This is also in analogy with the capacity of the penicillin binding component (PBC) to bind penicillin, existing within sensitive and resistant strains, as reported by *Cooper* (1956) The biochemical nature of PBC is as yet unknown but is suggested to be an enzyme, located in the membrane Recently *Roman & Eriksson* (1963), in studying *E coli*, have proposed

The author wishes to express thanks to *Dr B Norkrans* for her continued interest and advice in this work Thanks are also due to Professor *H Ryden* for placing at the author's disposal accommodation for the animals and to Kabi Ltd for a generous gift of streptomycinsulphate

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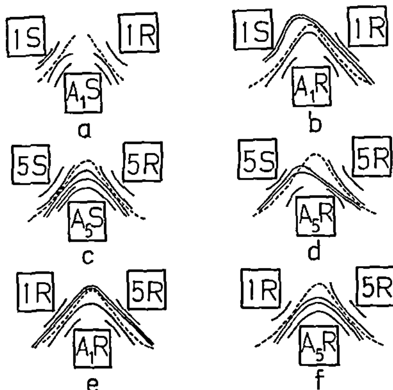


Fig 1

Precipitation patterns of the streptomycin sensitive strains 1 and 5 and their corresponding resistant strains against homologous and heterologous antisera

1S = sensitive strain 1
1R = resistant strain 1
A_1S = antiserum against 1S
A_1R = antiserum against 1R
5S = sensitive strain 5
5R = resistant strain 5
A_5S = antiserum against 5S
A_5R = antiserum against 5R

As seen from Fig 1, Plates a and b, the streptomycin sensitive strain 1 gave five precipitation lines against both the corresponding antiserum and A_1R The reaction, however, is more vigorous in the latter case As has been pointed out by *Oeding* (1960) the ability of an antigen to cause agglutination and to induce antibody formation varies from one staphylococcal strain to another The same may be valid also for precipitinogens In one strain the antigen may be a good precipitinogen but poorly antigenic, possibly due to the structural localization of the antigenic determinant The knowledge in this field, however, is rather scanty.

The resistant strain 1, giving six precipitation lines against the corresponding antiserum but only four ones against A_1S, shows in Plate b four shared antigenic determinants with the parent strain The quantitative difference between two of these shared antigenic factors as revealed by the location of the two common upper precipitation lines may be due to a difference in the extractibility of these two antigens

PBC to be a metabolite in a pathway for the synthesis of cell wall components

A comparative study between streptomycin resistant strains and their parent strains has been made by *Stern & Elek* (1957), using agglutination reactions with absorbed antisera against heat killed bacteria. Cowan's three types were used as test organisms. They found no evidence indicating changes in the antigenic structure of the resistant variants.

This paper presents the results obtained with antisera against formalin killed bacteria tested by the double diffusion agar technique with which minute differences can be detected.

MATERIALS AND METHODS

Organisms

Two coagulase positive strains of *Staphylococcus aureus* were chosen for this study, i.e. strain 5 and strain 1, corresponding to Cowan's type I. The strains have previously been examined and characterized (*Wahlstrom & Norkrans* 1963).

Preparation of Streptomycin Resistant Strains

Initially the resistant strains were prepared from one cell cultures of the sensitive strains by the gradient plate technique of *Szybalski (1952)*, followed by repeated transfers in liquid medium of increasing streptomycin concentrations. Streptomycin was dissolved in sterile phosphate buffer pH 8.0, which was also used as the diluent. The medium consisted of Antibiotic medium 3 (Bacto Penassay Broth Difco). The strains were made resistant to 163.84 mg of streptomycinsulphate/ml.

In examining the individual antisera from the rabbits before they were pooled a variation of ± 1 in the number of precipitation lines with antisera in the corresponding tests was observed, hence a difference of one in the number of precipitation lines given by two strains cannot be considered a criterion for a real difference in the antigenic pattern, as it can be caused by the variability in immune response of the different experimental animals. The following antisera were prepared: A_1S = antiserum against the sensitive strain 1 (1S), A_1R = antiserum against the resistant strain 1 (1R), A_5S = antiserum against the sensitive strain 5 (5S) and A_5R = antiserum against the resistant strain 5 (5R).

RESULTS AND DISCUSSION

The double diffusion agar technique provides a very useful tool in separating different antigen antibody systems and has been used in numerous investigations (For references see *Ouchterlony 1962*). In comparing systems like those in this study, where crude antigen solutions are used, some sort of standardization is imperative. One way of doing this is to select a reference band. If this is found to be caused by an antigenic determinant common to the antigen solutions tested, it should form a well recognized precipitation line with the corresponding antibody. This was found to be the case with the precipitation line in Fig. 1 marked by a dotted line. The density, time of developing and location of this reference band was found to remain constant when using an antigen dilution *1:10* against undiluted antisera.

strains of previous studies show qualitative changes in the antigenic pattern when compared with the parent strains Furthermore, it was not possible to find any accordance between the resistant strains, indicating different ways of acquiring this type of antibiotic resistance A penicillinase producing strain, however, did not show any changes The former are also supposed to develop a resistance of a more individual nature than that of penicillinase producing strains (*Knox & Smith* 1961)

SUMMARY

1 The antigenic pattern of two streptomycin resistant staphylococcal strains and their corresponding parent sensitive strains have been examined by means of the double diffusion agar technique

2 A quantitative change seems to exist in the same two antigens of the sensitive strains although no qualitative change could be found in the antigenic structure with the soluble antigenic extracts

3 The results have been discussed in connection with earlier results obtained with penicillin resistant strains

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from the corresponding strains The appearance of these two precipitation lines is dissimilar distinct between 1R–A_1R, but diffuse between 1S–A_1R However, in an analogous test with strain 5, Plate d, the same situation appears, though not as pronounced as in Plate b The lines here are quite distinct on both sides That it is the same two antigens which cause these precipitation lines is seen in the crosstests on Plates e and f Here it is also seen that the quantity of these antigens is the same in the two antigenic extracts of the resistant strains The location of the precipitation lines is different, in Plate e above and in Plate f under the "reference band", which can be explained as due to a difference in the amount of antibodies directed towards them

Nor did strain 5S reveal any qualitative changes in the antigenic pattern on acquiring streptomycin resistance Plate c shows seven precipitation lines between 5S–A_5S and six such lines between 5R–A_5S Four of them show deviation and complete fusion, indicating at least four antigenic determinants in common Plates c and d show the same qualitative antigenic pattern of the antigen solutions from the strains 5S and 5R against A_5S and A_5R, as the deviation of one precipitation line may be caused by the variation of the experimental animals (see above, p 109)

The great similarities in the antigenic pattern between the strains is probably explained by the fact that they belong to the same phage group (*Wahlstrom & Norkrans* 1963)

Data presented by *e g Demerec* (1948), indicate that the mechanism of development of streptomycin resistance is similar to that pertaining with penicillin resistance, and that mutations are responsible for the origin of drug resistance There is hardly any drug to which resistance emerges as readily as to streptomycin Several genes seem to be involved in both of these two types of antibiotic resistance In the case of penicillin resistance, all the genes have the same potency, but in streptomycin resistance they vary greatly A high degree of resistance is therefore possible in single-step resistant strains towards streptomycin The readiness with which the streptomycin resistant strains 1 and 5 developed, indicates them to be single-step mutants From earlier analogous studies wit penicillin resistant strains (*Norkrans & Wahlstrom* 1962, *Wahlstrom & Norkrans* 1963), there is no doubt that they acquired a high degree of resistance by a multi step process

In this study, limited only to a recording of the number of precipitation lines, there is no evidence of a qualitative change in the antigenic pattern of a sensitive strain on acquiring streptomycin resistance As has been discussed, it cannot be excluded, however, that a quantitative change does in fact occur The acquisition of streptomycin resistance, proposed to be caused by a single-step mutation, seems to be reflected in the same two common antigenic determinants of the strains tested, thus indicating a common resistance mechanism

On the other hand multi-step mutants such as the penicillin resistant

The indirect Proof

If two or more hypotheses could be used, and these hypotheses 1) exclude each other and 2) are all hypotheses possible, one of these hypotheses may sometimes be proved indirectly, namely if the other hypotheses can be rejected using usual significance tests As a further consequence the hypothesis proved by this method does not need to say anything about the character or distribution of the observations, whereas this is necessary for the hypotheses to be rejected

Significance tests of usual type t χ^2, v² etc (see for instance *Fisher & Yates* (6)) are in a way indirect proofs it is rejected, that two groups are identic and then they must be different These tests are frequently used in biological work, but the employment of the indirect proof mentioned above is rarely met

Two examples will be quoted

A) In the discussion about the origin of staphylococcal infection the possibility of self infection has played an important role since *Danholt* (4) reported 24 cases of cutaneous infection in patients, who carried the infective type in their nose (carriers) It has been stressed in support of this hypothesis, that infections are more frequent in carriers than in non carriers, 7 and 2 per cent respectively (*Williams et al* (10)), and that the patients were carriers before infection occurred

These statements are certainly in accordance with the hypothesis of self infection and if they were not the hypothesis had already been rejectable But it is not surprising, that carriers are susceptible to infection, both conditions supposedly being determined by the resistance of the patients And the infected patients might by chance be infected from outside by the type of *Staphylococcus aureus* already resident in their nose And this is the alternative hypothesis

Do nasal carriers of *Staphylococcus aureus* get their wounds infected with staphylococcal strains from other persons and not from their own nose?

To try this hypothesis it would be necessary to know the carriers and clinically infected persons among other patients, nurses and doctors In each case of infection the probability that the patient had by chance acquired the nasal type from outside could be estimated from the number of persons harbouring the incriminated type as numerator, and the total number of carriers and septic persons as denominator

This could be done in an investigation carried out 1957–59 at Rigshospitalet in Copenhagen (*Siboni* (8)) 37 of the infected patients were nasal carriers before the infection occurred Among these 37 patients 28 became infected with the type of *Staphylococcus aureus* already inhabiting their nostrils If the presence of the nasal strain of *Staphylococcus aureus* in the wound had been determined by chance contraction from outside this would have happened in only 5.5 cases The difference between these figures is highly significant (t = 5.3), and self-

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ON ARGUMENTATION IN BIOLOGY

The Indirect Proof

By

KNUD E SIBONI

Received 6 ii 64

In biology including medicine as in other empiric sciences argumentation cannot be exclusively deductive The elements of deduction are found in Euclid's geometry, which leaves no doubt that the triangles are really congruous

In the empiric sciences a hypothesis is deduced from some premises because the investigator in a set of observations has observed a certain trend New sets of observations are necessary to decide if the hypothesis may be correct Disagreement between the new observations and the hypothesis results in the rejection of the hypothesis The evaluation of this disagreement is statistical one of the usual significance tests gives the probability to find the given set of observations if the hypothesis is true If this probability is sufficiently small the hypothesis is rejected The criterion for rejection is, therefore, quantitative and more or less rigorous, most often a probability of 1 or 5 per cent for deviation by chance

If the probability is larger the hypothesis is not rejected In this situation it is often concluded, that the hypothesis has been proved, in *analogy to the congruous triangles But this is not necessarily true,* for using the premises one could most often construct several hypotheses agreeing with the observations done *Green* (7) wrote "A sufficiently ingenious mechanic could separate the parts of a baby Austin and use them to make a perambulator or a pressure pump or a hair dryer of sorts If the mechanic was not particularly bright and was informed as to the source of these parts he might be tempted into believing, that they were in fact designed for the particular end he happened to have in view"

Fisher (5) formulates the agreement as the "null hypothesis" and writes "it should be noted, that the null hypothesis is never proved or established but it is possibly disproved in the course of experimentation"

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The indirect Proof

If two or more hypotheses could be used and these hypotheses 1) exclude each other and 2) are all hypotheses possible one of these hypotheses may sometimes be proved indirectly namely if the other hypotheses can be rejected using usual significance tests. As a further consequence the hypothesis proved by this method does not need to say anything about the character or distribution of the observations where as this is necessary for the hypotheses to be rejected.

Significance tests of usual type t χ^2 v^2 etc (see for instance *Fisher & Yates* (6)) are in a way indirect proofs: it is rejected that two groups are identic and then they must be different. These tests are frequently used in biological work but the employment of the indirect proof mentioned above is rarely met.

Two examples will be quoted.

A) In the discussion about the origin of staphylococcal infection the possibility of self infection has played an important role since *Danholt* (4) reported 24 cases of cutaneous infection in patients who carried the infective type in their nose (carriers). It has been stressed in support of this hypothesis that infections are more frequent in carriers than in non carriers 7 and 2 per cent respectively (*Williams et al* (10)) and that the patients were carriers before infection occurred.

These statements are certainly in accordance with the hypothesis of self infection and if they were not the hypothesis had already been rejectable. But it is not surprising that carriers are susceptible to infection both conditions supposedly being determined by the resistance of the patients. And the infected patients might by chance be infected from outside by the type of *Staphylococcus aureus* already resident in their nose. And this is the alternative hypothesis:

Do nasal carriers of *Staphylococcus aureus* get their wounds infected with staphylococcal strains from other persons and not from their own nose?

To try this hypothesis it would be necessary to know the carriers and clinically infected persons among other patients, nurses and doctors. In each case of infection the probability that the patient had by chance acquired the nasal type from outside could be estimated from the number of persons harbouring the incriminated type as numerator, and the total number of carriers and septic persons as denominator.

This could be done in an investigation carried out 1957 59 at Rigshospitalet in Copenhagen (*Sibom* (8)). 37 of the infected patients were nasal carriers before the infection occurred. Among these 37 patients 28 became infected with the type of *Staphylococcus aureus* already inhabiting their nostrils. If the presence of the nasal strain of *Staphylococcus aureus* in the wound had been determined by chance contraction from outside this would have happened in only 5 5 cases. The difference between these figures is highly significant ($t = 5\ 3$), and self

infection must have occurred in approximately 22 out of the 37 cases (28 − 5 5)

B In the evaluation of blood cultured for bacteria contamination causes great difficulty as its frequency amounts to 5–15 per cent in different series, i e of the same size or even more frequent than growth due to bacteraemia in the patients

When *Staphylococcus aureus* (coagulase positive) is found, the reason is most often a real bacteraemia, and the patients are severely ill, but *Staphylococcus aureus* is widely distributed among carriers and has a fair chance to be a contaminant It is, therefore, important to get a criterion for growth on account of bacteraemia

In an investigation at Blegdamshospitalet in Copenhagen (*Siboni* (9)) *blood cultures were normally done at least two times from each patient* In 1470 blood cultures from about 500 patients *Staphylococcus aureus* was found 91 times, which gives an average frequency per blood culture of 0 062 In 15 patients *Staphylococcus aureus* was found at least two times in subsequent cultures, in 8 patients in one of two subsequent blood cultures and in 2 patients once in one blood culture

The hypothesis alternative to bacteraemia is contamination

In order to see the distribution of a contaminant, *Staphylococcus albus* may be examined, as *Staphylococcus albus* judged from the clinical state of the patients in no case of this series was the cause of disease *Staphylococcus albus* is here defined as a Gram positive coccus growing in clusters and coagulase negative It was found 122 times, which gives an average frequency per blood culture of 0 083 In 10 patients *Staphylococcus albus* was found at least two times in subsequent cultures, in 66 patients in one of two subsequent blood cultures and in 25 in a single culture

TABLE 1

Distribution of Staphylococcus aureus and Staphylococcus albus among 1470 Blood Cultures from 500 Patients

	2 subsequent cultures positive	1 positive of 2 subsequent cultures	1 single culture positive
Staphylococcus aureus	15	8	2
Staphylococcus albus	10	66	25

It is seen that the distribution of *Staphylococcus aureus* is entirely different from that of *Staphylococcus albus*, *even if the last column of* Table 1 is excluded ($\chi^2 = 25.5$, $f = 1$, $P < 0.05$), and it may be concluded that *Staphylococcus aureus* was not distributed as the contaminant *Staphylococcus albus* A distribution at random among the blood cultures of a bacterium found with a frequency averaging 0 083 would 3–4 times give growth in both of two subsequent cultures and 76 times

in one of two These figures correspond closely to those of *Staphylococcus albus*

If the 8 cases where *Staphylococcus aureus* was found in one of two subsequent cultures were to be regarded as a result of contamination, the frequency, p of this contamination per blood culture would be determined by $2p(1-p)$ 500 = 8 hence p = 0 008 and this would be the maximal frequency for *Staphylococcus aureus* as a contaminant The probability to find *Staphylococcus aureus* in two subsequent cultures on account of contamination would be $0\ 008^2$ = 0 000064 and growth would appear in 0 032 among 500 patients Actually *Staphylococcus aureus* was found in 15 cases in at least two subsequent cultures

Conclusion *Staphylococcus aureus* does not occur in blood cultures as a randomly distributed contaminant *i e* when found it is normally a cause of disease The criterion for bacteraemia also appeared growth of *Staphylococcus aureus* in two subsequent bloodcultures means bacteraemia as this could be due to contamination only once among 15 625 pairs of blood cultures

DISCUSSION

In example B blood cultures the alternative hypothesis of contamination in a way expresses the error of the method It is well known from the analysis of variance to subtract this error from the total variance in order to determine the part of the variance caused by the investigated variable itself But the mere fact, that this variable exists and has a variance normally does not prove any hypothesis about it which is the case here And in example A self infection or not, the alternative hypothesis does not express the error, it is only a negation of the original hypothesis

It is a consequence of the quantitative character of significance tests that the alternative hypothesis cannot be rejected as a whole The alternative hypothesis may be correct as regards some of the observations But the rejection means that the alternative hypothesis does not satisfy the total set of observations and that the main hypothesis is correct at least concerning a part of the observations The size of this part can be estimated as done in example A

An important condition for the use of the indirect proof is certainly that the alternative hypothesis is correctly laid down If not its rejection is of no value and the whole proof is useless In example A it may be discussed if it is correct to use the total number of carriers and patients with septic lesions as the denominator in the estimate of the probability to get the nasal type by chance from outside as all types might not be infectious to the same degree and some of them perhaps not at all This could be compensated and also the infections were caused by a diversity of types

In example B it could be asked how one would know if the occurrence

infection must have occurred in approximately 22 out of the 37 cases (28 – 5 5)

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IMMUNOCHEMICAL STUDIES ON ANTIGEN PREPARATIONS FROM STAPHYLOCOCCUS AUREUS

2 *Precipitating and Erythrocyte Sensitizing Properties of Protein A (Antigen A) and Related Substances*

By

PER OEDING ARNE GROV and BERIT MYKLESTAD

Received 10 i 64

Antigen A of *Staphylococcus aureus* has been isolated in a relatively pure state by us and characterized chemically (2) While *Jensen* (9 10 11) considered the antigen to be a polysaccharide we in agreement with *Lofkvist & Sjoquist* (14) found that it is a protein which is probably identical to the protein fraction B described by *Verwey* in 1940 (23) We proposed to call this antigen *protein A* to avoid confusion with polysaccharide A and antigen *a*

Jensen et al (11) demonstrated that their extract A contained at least three different components 1 Antigen A characterized by its precipitation line on agar with normal human sera 2 Toxin D 3 A heterogenetic antigen of the Rantz type

Erythrocyte-sensitizing antigens have also been demonstrated in extracts of staphylococci by other authors (2? 21 19 17 5 15)

The intention of the present investigation has been to examine the serological properties of our purified protein A preparation As it was shown to contain in addition to its precipitinogen an antigen that sensitized tanned erythrocytes it was decided to examine the presence in extract A and polysaccharide A of sensitizing substances and to attempt to correlate our findings with earlier reports

MATERIALS AND METHODS

Strains

The Staph aureus strains used belong to our typing set (8) Strain Cowan I (NCTC 8530 was used to prepare extract A and a

of *Staphylococcus albus* in two subsequent blood cultures was caused by bacteriaemia. The answer is that *Staphylococcus albus* defined as here is a very heterogeneous group of the Micrococcaceae which ought to be divided using the criteria in *Bergey's* Manual of Determinative Bacteriology (2) or as *Baird Parker* did it (1). A division of this kind combined with sensitivity tests would yield numerous types of Staphylococcus albus making the probability to refind the same type very small.

The qualitative character of epidemiology perhaps makes it especially accessible to the use of the indirect proof but it could also be used within other parts of microbiology such as in the distinction between mutation and adaptation if the consequences of one of the two hypotheses were rejectable (*Burnet* (3)).

The indirect proof has supposedly been used within other fields of the natural sciences.

SUMMARY

It is mentioned that the accordance of a set of observations with the hypothesis is no proof for the correctness of this hypothesis while disagreement between observations and hypothesis disproves the latter.

Under special circumstances it is nevertheless possible to prove a hypothesis indirectly, namely when there are only one or a few alternative hypotheses and these can be rejected. (The indirect proof).

Two examples are discussed.

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The qualitative character of epidemiology perhaps makes it especially accessible to the use of the indirect proof, but it could also be used within other parts of microbiology such as in the distinction between mutation and adaptation, if the consequences of one of the two hypotheses were rejectable (*Burnet* (3))

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human serum (see Fig 2 in *Grov et al* (2)) The other line, appearing only in Cowan I antiserum was stronger and sharper than the first one *This line could not* be identified with any other known staphylococcal antigen

As the double line appeared regularly in all our Cowan I antisera and was found with extracts of other *Staph aureus* strains than Cowan I, it did not seem to be artificial However, we did not succeed in separating the two lines on ion exchange columns on electrophoresis, or on immunoelectrophoresis Both disappeared when the material was digested with trypsin

Sensitization of Normal Sheep Cells

When 0 1 ml of packed sheep cells were treated with 0 2 mg of extract A the cells were sensitized to *agglutination in* Cowan I antiserum but not in a pool of normal human sera or in 10 sera from blood donors The haemagglutinin titre in Cowan I antiserum was 1 1,280 When washings from agar cultures or crude extracts were used all the *Staph aureus* strains examined had the ability to sensitize normal sheep cells, and haemagglutinins were found in the rabbit immune sera (Table 1) The strains seemed to contain varying amounts of the antigen The two *Staph epidermidis* strains and the two *B subtilis* strains examined, but not the one strain of *M lysodeikticus*, also sensitized normal sheep cells *to agglutination in Cowan I antiserum*

However the ability to sensitize normal sheep erythrocytes was not demonstrated in the purified protein A, *in polysaccharide A, in polysaccharide* B or AC isolated from *Staph epidermidis* (12), or in preparations of *Staph aureus* wall teichoic acid containing both α- and β N acetylglucosamine residues

TABLE 1
Haemagglutination of SNSC

Materials used for sensitization	Haemagglutinin titres (reciprocal values) in Cowan I serum
Staph aureus Cowan I crude extract	1 280
" " Cowan I extract A	1 280
" " Cowan I [illegible]	–
[illegible]	640
[illegible]	–
[illegible]	–
[illegible]	640
[illegible]	2 560
[illegible]	–
[illegible] washings	1.280
" 1254 [illegible] B	–
B subtilis 10865 washings	2 560
" [illegible] washings	1.280
M lysodeikticus 2665 washings	–

SNSC = Sensitized normal sheep cells

Antigenic Materials

Purified protein A was prepared according to the method used by us (2) and extract A (crude protein A) according to *Jensen's* procedure (10). Polysaccharide A was obtained using the purification procedure of *Haukenes* (3). Polysaccharide B and polysaccharide AC were prepared according to the method of *Losnegard & Oeding* (12). Washings from agar cultures and crude extracts were prepared from a number of the strains mentioned above.

Teichoic acid samples from *Staph. aureus* cell walls were kindly furnished by Professor *J. Baddiley*, Newcastle, and by Professor *J. L. Strominger*, St. Louis.

Immune Sera

Rabbit immune sera against strains Cowan I, Wood 46 and other *Staph. aureus* type strains, and against the four *Staph. epidermidis* strains were produced by intravenous injections of formalin killed bacteria as described by *Oeding* (18). The same pool of 10 normal human sera was used in all experiments. In addition individual sera from blood donors were examined.

Immune serum against the antigen in polysaccharide A which sensitizes tanned sheep cells, was prepared in the following way: Tanned sheep erythrocytes were sensitized with polysaccharide A, washed three times with saline, and made up to a 10 per cent solution in saline. Rabbits were given three series of intravenous injections, each series consisting of three injections. The injections were given daily with 5 days' interval between the series. Each dose contained 1 ml of erythrocyte solution. Before use the serum was absorbed with normal sheep cells.

Methods

The ring test precipitation was carried out according to the method used by *Haukenes et al.* (7) and the agar precipitation was that used by *Haukenes & Oeding* (8).

A 1:40 000 solution of tannic acid was used for the preparation of sheep cells. The sensitization of normal and tanned sheep cells was performed essentially as described by *Morse* (15). Usually 0.1 ml of packed erythrocytes were sensitized with 0.2 mg of antigenic material. The sera were diluted in tubes to a total volume of 0.5 ml. To each tube was added 0.1 ml of a 1.25 per cent solution of the sensitized cells. After thorough mixing the tubes were placed in a water bath at 37°C for 30 minutes and then at room temperature over night. The haemagglutination was recorded before and after light shaking of the tubes.

Absorption of sensitizing antigen from the preparations was performed with large doses of normal or tanned sheep cells in a 37° C water bath for 30 minutes. The complete removal of sensitizing antigen was controlled by testing the sensitizing ability of the absorbed preparation and the haemagglutinating ability of the cells used for absorption.

Absorption of antibodies against the sensitizing antigens was performed by adding a surplus of sensitized normal or tanned sheep cells to the serum diluted 1:2 and incubating at 37° C for 30 minutes. Complete absorption was controlled by testing the sensitized cells used for absorption and the haemagglutinating ability of the absorbed serum.

RESULTS

Precipitation

The purification of protein A on DEAE cellulose column led to an increasing ring test titre of the material. Whereas the ring test titre of extract A in a concentration of 1 mg/ml was only 1:10 with Cowan I antiserum, the titre of the purified protein A was 1:80.

On agar precipitation both protein A and extract A gave two closely situated lines with Cowan I antiserum but only a single line with normal human serum. Other lines were not observed. *The lines of the two* products showed reactions of identity. The line closest to the antigen well gave a reaction of identity with the single line appearing with the

showed that it contained 5 amino acids (Lys, Gly, Ser, Glu A, and Ala) and no sugar. The sensitizing ability was destroyed by trypsin (Table 4). These experiments thus confirm the results of the absorptions in that the antigen in extract A (and protein A) sensitizing tanned sheep cells is a distinct antigen. The antigen is of protein nature.

TABLE 2

Haemagglutination in Rabbit Immune Sera and Human Serum

Sensitized sheep cells	Haemagglutinin titres (reciprocal values) in sera			
	Cowan I	Wood 46	Poly A STSC*	Human
Extract A STSC	2 560	80	40	1 280
Extract A SNSC	1 280	160	–	–
Protein A STSC	20 480	80	40	10 240
Poly A STSC	80	20 480	80	640

STSC = Sensitized tanned sheep cells
SNSC = Sensitized normal sheep cells
* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells

TABLE 3

Absorption of Preparations with Tanned Sheep Cells

Preparations		Precipitation in homologous serum* (reciprocal values)		Haemagglutination in homologous serum* (reciprocal values)	
		Ring test	Agar	STSC	SNSC
Poly A	Not absorbed	3 000	+	20 480	–
	Absorbed	3 000	+	–	
Protein A	Not absorbed	80	+	20 480	–
	Absorbed	80	+		
Extract A	Not absorbed	10	+	2 560	1 280
	Absorbed	10	+	–	320

STSC = Sensitized tanned sheep cells
SNSC = Sensitized normal sheep cells
* Polysaccharide A examined in Wood 46 serum, protein A and extract A in Cowan I serum

Our polysaccharide A preparation also sensitized tanned sheep cells (Table 2). Complete sensitization of 0.1 ml of packed sheep cells was obtained with 0.02 mg of polysaccharide A. The haemagglutinin titre in Wood 46 antiserum was 1:20 480 using standard conditions and even 1:40 960 when the erythrocyte concentration was [illegible]

[illegible]

[illegible] in strain Cowan I, from which protein A is prepared

The ability to sensitize normal sheep cells was destroyed at 100° C at alkaline pH but not at acid pH It was resistant to trypsin (Table 4) These properties and the distribution of the antigen indicate that it is of the Rantz type (21)

The antigen sensitizing normal sheep cells could be separated from the precipitinogens protein A and polysaccharide A on ion exchange fractionation of crude extracts An experiment was described in part 1 of this investigation (2), in which chromatography of extract A resulted in the separation of 3 fractions One fraction contained only the antigen sensitizing normal sheep cells, the second fraction contained only an antigen sensitizing tanned sheep cells (see below), and the third fraction contained the precipitinogen (protein A) in addition to minor amounts of the two sensitizing substances Chemical examination of the fraction containing only the antigen sensitizing normal sheep cells showed that it contained ribose, glucuronic acid, and only small amounts of three amino acids

Absorption of extract A with normal sheep cells, removed all antigen sensitizing normal sheep cells while the precipitin titre (protein A) and the ability to sensitize tanned sheep cells remained unchanged The absorption experiments and the separation on DEAE cellulose column thus show that the antigen sensitizing normal sheep cells is not identical to any other antigen present

Sensitization of Tanned Sheep Cells

Extract A and protein A sensitized tanned sheep cells to agglutination both in Cowan I antiserum and in normal human serum When 0 1 ml of packed, tanned erythrocytes were treated with 0 2 mg of protein A (or extract A), the haemagglutinin titre of Cowan I antiserum was 1 20,480 to 1 2,560 and that of normal human serum of the same order (Table 2) When extract A was absorbed with large doses of tanned erythrocytes, all antigen sensitizing tanned cells was removed while the antigen sensitizing normal cells and the precipitinogen were still present (Table 3) Absorption of Cowan I antiserum with tanned erythrocytes sensitized with extract A, removed the corresponding antibodies, but not antibodies against normal erythrocytes sensitized with extract A or against the precipitinogen Thus the antigen sensitizing tanned sheep cells is not identical to the antigen in extract A which sensitizes normal cells, or to the precipitinogen (protein A) The antigen sensitizing tanned cells demonstrated in extract A and in protein A were shown by absorption to be identical

In the fractionation experiment on DEAE cellulose column reported in (2), the material sensitizing tanned cells could be separated from the two other antigens present in extract A The separated material was only able to sensitize tanned sheep cells, and gave no precipitation on the ring test or on agar The chemical examination of the fraction

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Poly A STSC	80	20 480	80	640

STSC = Sensitized tanned sheep cells
SNSC = Sensitized normal sheep cells
* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells

TABLE 3

Absorption of Preparations with Tanned Sheep Cells

Preparations		Precipitation in homologous serum* (reciprocal values)		Haemagglutination in homologous serum* (reciprocal values)	
		Ring test	Agar	STSC	SNSC
Poly A	Not absorbed	3 000	+	20 480	–
	Absorbed	3 000	+	–	
Protein A	Not absorbed	80	+	20 480	–
	Absorbed	80	+		
Extract A	Not absorbed	10	+	2 560	1,280
	Absorbed	10	+	–	320

STSC = Sensitized tanned sheep cells
SNSC = Sensitized normal sheep cells
* Polysaccharide A examined in Wood 46 serum, protein A and extract A in Cowan I serum

Our polysaccharide A preparation also sensitized tanned sheep cells (*Table 2*). Complete sensitization of 0.1 ml of packed sheep cells was obtained with *0.02 mg of* polysaccharide A. The haemagglutinin titre in Wood 46 antiserum was 1:20,480 using standard conditions and even 1:40,960 when the erythrocyte concentration was diminished [illegible] Wood 46 and [illegible] tanned sheep [illegible] in *Wood 46* antiserum was also demonstrated in other strains of *Staph. aureus*, *e.g.* in strain Cowan I, from which protein A is prepared.

The ability to sensitize normal sheep cells was destroyed at 100° C at alkaline pH but not at acid pH It was resistant to trypsin (Table 4) These properties and the distribution of the antigen indicate that it is of the Rantz type (21)

The antigen sensitizing normal sheep cells could be separated from the precipitinogens protein A and polysaccharide A on ion exchange fractionation of crude extracts An experiment was described in part I of this investigation (2), in which chromatography of extract A resulted in the separation of 3 fractions One fraction contained only the antigen sensitizing normal sheep cells, the second fraction contained only an antigen sensitizing tanned sheep cells (see below), and the third fraction contained the precipitinogen (protein A) in addition to minor amounts of the two sensitizing substances Chemical examination of the fraction containing only the antigen sensitizing normal sheep cells, showed that it contained ribose, glucuronic acid, and only small amounts of three amino acids

Absorption of extract A with normal sheep cells, removed all antigen sensitizing normal sheep cells while the precipitin titre (protein A) and the ability to sensitize tanned sheep cells remained unchanged The absorption experiments and the separation on DEAE cellulose column thus show that the antigen sensitizing normal sheep cells is not identical to any other antigen present

Sensitization of Tanned Sheep Cells

Extract A and protein A sensitized tanned sheep cells to agglutination both in Cowan I antiserum and in normal human serum When 0 1 ml of packed, tanned erythrocytes were treated with 0 2 mg of protein A (or extract A), the haemagglutinin titre of Cowan I antiserum was 1 20,480 to 1 2,560 and that of normal human serum of the same order (Table 2) When extract A was absorbed with large doses of tanned erythrocytes, all antigen sensitizing tanned cells was removed while the antigen sensitizing normal cells and the precipitinogen were still present (Table 3) Absorption of Cowan I antiserum with tanned erythrocytes sensitized with extract A, removed the corresponding antibodies, but not antibodies against normal erythrocytes sensitized with extract A or against the precipitinogen Thus the antigen sensitizing tanned sheep cells is not identical to the antigen in extract A which sensitizes normal cells, or to the precipitinogen (protein A) The antigen sensitizing tanned cells demonstrated in extract A and in protein A, were shown by absorption to be identical

In the fractionation experiment on DEAE cellulose column reported in (2), the material sensitizing tanned cells could be separated from the two other antigens present in extract A The separated material was only able to sensitize tanned sheep cells, and gave no precipitation on the ring test or on agar The chemical examination of the fraction

showed that it contained 5 amino acids (Lys, Gly, Ser, Glu A, and Ala) and no sugar The sensitizing ability was destroyed by trypsin (Table 4) These experiments thus confirm the results of the absorptions in that the antigen in extract A (and protein A) sensitizing tanned sheep cells is a distinct antigen The antigen is of protein nature

TABLE 2

Haemagglutination in Rabbit Immune Sera and Human Serum

Sensitized sheep cells	Haemagglutination titres (reciprocal values) in sera			
	Cowan I	Wood 46	Poly A STSC*	Human
Extract A STSC	2 560	80	40	1 280
Extract A SNSC	1 280	160	–	–
Protein A STSC	20 480	80	40	10 240
Poly A STSC	80	20 480	80	640

STSC = Sensitized tanned sheep cells
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STSC = Sensitized tanned [illegible]
SNSC = [illegible]
* *Polysa*[illegible]
Cowan I

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possible to separate the antigen sensitizing tanned cells from the mixture of sensitizing and precipitating substances. In the fraction containing the sensitizing antigen the same 5 amino acids were found as in the corresponding fraction obtained from extract A. The same amino acids were also demonstrated by *Haukenes* (4) in polysaccharide A, constituting the mucopeptide moiety of the material. The antigen in polysaccharide A sensitizing tanned cells was sensitive to trypsin, whereas the precipitinogen (polysaccharide A) is resistant.

TABLE 5

Haemagglutination after Cross Absorptions of Sera with Polysaccharide A STSC and Protein A STSC

Sensitized sheep cells	Cowan I serum absorbed with		Wood 46 serum absorbed with		Poly A STSC serum* absorbed with	
	Poly A STSC	Protein A STSC	Poly A STSC	Protein A STSC	Poly A STSC	Protein A STSC
Poly A STSC		1:80		1:80	–	1:40
Protein A STSC		–	–	–		

STSC = Sensitized tanned sheep cells
* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells
Serum titres before absorption are given in Table 2

A sample of polysaccharide A was absorbed with tanned sheep cells until the haemagglutinin reaction became negative. The supernatant was then analyzed for the presence of amino acids. The small amount of material made it difficult to obtain exact results, but it seemed that the amino acid content had been considerably reduced after absorption.

The tanned cell sensitizing antigens contaminating our protein A and polysaccharide A preparations can thus be separated from the precipitinogens. The latter have not yet been prepared free of the sensitizing antigens. As these sensitizing antigens are proteins, the polysaccharide A precipitinogen may be prepared free of the sensitizing antigen by enzymatic digestion. The protein A precipitinogen is, however, destroyed by trypsin.

In polysaccharide A the sensitizing antigen is undoubtedly attached to the mucopeptide moiety. *Staph aureus* H ribitol teichoic acid has the same precipitating antigen as polysaccharide A but lacks the mucopeptide group (6). None of the *Staph aureus* ribitol teichoic acids examined were able to sensitize tanned sheep cells.

DISCUSSION

The significance of the double line appearing on agar precipitation of protein A against the homologous whole rabbit immune serum but not against normal human serum has not been explained. It appears quite regularly and does not seem to be artificial. The double line has earlier

TABLE 4
Tryptic Digestion of Preparations

Preparations		Precipitation in homologous serum*		Haemagglutination in Cowan I serum	
		Ring test	Agar	STSC	SNSC
Poly A	Not treated	+	+	+	–
	Treated	+	+	–	
Protein A	Not treated	+	+	+	–
	Treated	–	–	–	
Extract A	Not treated	+	+	+	+
	Treated	–	–	–	+

STSC = Sensitized tanned sheep cells
SNSC = Sensitized normal sheep cells
* Polysaccharide A precipitated in Wood 46 serum protein A and extract A in Cowan I serum
\+ after tryptic digestion = titres unchanged

Haemagglutinins reacting with polysaccharide A sensitized tanned sheep cells were present in varying amounts in all our *Staph aureus* antisera, including serum Cowan I, and in normal human serum These haemagglutinins were also present in antisera against 3 of the 4 strains of *Staph epidermidis* from which polysaccharide B and AC were prepared The strains had been shown to contain small amounts of polysaccharide A (13)

Absorption of polysaccharide A with tanned sheep cells showed that its sensitizing antigen is not identical to the precipitinogen The sensitizing substance could be completely removed, whereas the ring test titre and the precipitation line on agar remained unchanged (Table 3)

Cross-absorption experiments were performed to see whether the substance in protein A sensitizing tanned sheep cells is identical to that in polysaccharide A As shown in Table 2, the two substances cross-react but the titres are much higher in the homologous sera than in the heterologous sera The cross-absorptions (Table 5) showed that polysaccharide A sensitized tanned cells were able to exhaust the sera, whereas protein A sensitized tanned cells did not seem to remove the antibodies directed towards polysaccharide A sensitized tanned cells The two substances sensitizing tanned sheep cells thus seem to be different serologically although they appear to be very similar chemically This problem will be further investigated

Rabbits were immunized with tanned sheep erythrocytes sensitized with polysaccharide A After absorption of the sera with normal sheep cells, they agglutinated both polysaccharide A sensitized tanned cells and protein A sensitized tanned cells to low titres (Table 2) Precipitins against polysaccharide A or agglutinins toward whole, live staphylococci were not detected

When polysaccharide A was run on a DEAE cellulose column, it was

possible to separate the antigen sensitizing tanned cells from the mixture of sensitizing and precipitating substances. In the fraction containing the sensitizing antigen, the same 5 amino acids were found as in the corresponding fraction obtained from extract A. The same amino acids were also demonstrated by *Haukenes* (4) in polysaccharide A, constituting the mucopeptide moiety of the material. The antigen in polysaccharide A sensitizing tanned cells, was sensitive to trypsin, whereas the precipitinogen (polysaccharide A) is resistant.

TABLE 5

Haemagglutination after Cross Absorptions of Sera with Polysaccharide A STSC and Protein A STSC

Sensitized sheep cells	Cowan I serum absorbed with		Wood 46 serum absorbed with		Poly A STSC serum* absorbed with	
	Poly A STSC	Protein A STSC	Poly A STSC	Protein A STSC	Poly A STSC	Protein A STSC
Poly A STSC	–	1 80	–	1 80		1 40
Protein A STSC	–	–	–	–		–

STSC = Sensitized tanned sheep cells

* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells. Serum titres before absorption are given in Table 2.

A sample of polysaccharide A was absorbed with tanned sheep cells until the haemagglutinin reaction became negative. The supernatant was then [illegible]

The tanned cell sensitizing antigens contaminating our protein A and polysaccharide A preparations can thus be separated from the precipitinogens. The latter have not yet been prepared free of the sensitizing antigens. As these sensitizing antigens are proteins, the polysaccharide A precipitinogen may be prepared free of the sensitizing antigen by enzymatic digestion. The protein A precipitinogen is, however, destroyed by trypsin.

In polysaccharide A the sensitizing antigen is undoubtedly attached to the mucopeptide moiety. *Staph. aureus* H ribitol teichoic acid has the same precipitating antigen as polysaccharide A but lacks the mucopeptide group (6). None of the *Staph. aureus* ribitol teichoic acids examined were able to sensitize tanned sheep cells.

DISCUSSION

The significance of the double line appearing on agar precipitation of protein A against the homologous whole, rabbit imm[illegible]

against [illegible]

regul[illegible]

TABLE 4
Tryptic Digestion of Preparations

Preparations		Precipitation in homologous serum*		Haemagglutination in Cowan I serum	
		Ring test	Agar	STSC	SNSC
Poly A	Not treated	+	+	+	–
	Treated	+	+	–	
Protein A	Not treated	+	+	+	–
	Treated	–	–	–	
Extract A	Not treated	+	+	+	+
	Treated	–	–	–	+

STSC = Sensitized tanned sheep cells
SNSC = Sensitized normal sheep cells
* Polysaccharide A precipitated in Wood 46 serum protein A and extract A in Cowan I serum
+ after tryptic digestion = titres unchanged

Haemagglutinins reacting with polysaccharide A sensitized tanned sheep cells were present in varying amounts in all our *Staph aureus* antisera, including serum Cowan I, and in normal human serum These haemagglutinins were also present in antisera against 3 of the 4 strains of *Staph epidermidis* from which polysaccharide B and AC were prepared The strains had been shown to contain small amounts of polysaccharide A (13)

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When polysaccharide A was run on a DEAE cellulose column, it was

possible to separate the antigen sensitizing tanned cells from the mixture of sensitizing and precipitating substances In the fraction containing the sensitizing antigen, the same 5 amino acids were found as in the corresponding fraction obtained from extract A The same amino acids were also demonstrated by *Haukenes* (4) in polysaccharide A constituting the mucopeptide moiety of the material The antigen in polysaccharide A sensitizing tanned cells was sensitive to trypsin, whereas the precipitinogen (polysaccharide A) is resistant

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	Poly A STSC	Protein A STSC	Poly A STSC	Protein A STSC	Poly A STSC	Protein A STSC
Poly A STSC		1 80		1 80		1 40
Protein A STSC	–	–			–	–

STSC — Sensitized tanned sheep cells

* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells Serum titres before absorption are given in Table 2

A sample of polysaccharide A was absorbed with tanned sheep cells until the haemagglutinin reaction became negative The supernatant was then analyzed for the presence of amino acids The small amount of material made it difficult to obtain exact results but it *seemed* that the amino acid content had been considerably reduced after absorption

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DISCUSSION

The significance of the double line appearing on agar precipitation of Protein A against the homologous whole rabbit imm[illegible]

against [illegible]

Figu[illegible]

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Tryptic Digestion of Preparations

Preparations		Precipitation in homologous serum*		Haemagglutination in Cowan I serum	
		Ring test	Agar	STSC	SNSC
Poly A	Not treated	+	+	+	–
	Treated	+	+	–	
Protein A	Not treated	+	+	+	–
	Treated	–	–	–	
Extract A	Not treated	+	+	+	+
	Treated	–	–	–	+

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When polysaccharide A was run on a DEAE cellulose column, it was

sensitizing tanned cells can be separated from the precipitinogen on the ion exchange column it seems to be quite firmly attached to the teichoic acid The sensitizing antigen may therefore have the same distribution as polysaccharide A (teichoic acid) i e be present in practically all *Staph aureus* strains This may explain why the sensitizing antigen has also been demonstrated in certain strains of *Staph epidermidis* which according to *Losnegard & Oeding* (13) in addition to polysaccharide B and C also have small amounts of polysaccharide A The distribution in staphylococci of antigens sensitizing tanned erythrocytes should be more thoroughly investigated

Morse (15) reported that the teichoic acid isolated by him from the wall of one strain of *Staph aureus* sensitized tanned sheep cells and that β linked N acetylglucosamine was responsible for this activity We found that the specific line on agar given by *Staph aureus* H teichoic acid and polysaccharide A was due to the β linked N acetylglucosamine (6) The teichoic acid preparations examined by us were however not able to sensitize erythrocytes Further our tanned cells sensitizing substances was separated from the precipitinogen on the ion exchange column and shown to be composed of 5 amino acids *Morse* used a 100 times higher sensitizing dose than we did and it is probably that the sensitizing ability of his teichoic acid preparation is due to contamination with the sensitizing protein antigen

SUMMARY

Purified protein A (antigen A) has been examined serologically On agar precipitation a single line developed with normal human serum whereas a double line was regularly observed with rabbit immune serum

An antigen sensitizing tanned sheep erythrocytes to agglutination in rabbit immune serum and in normal human serum was demonstrated in protein A A similar antigen was present in polysaccharide A but not in wall teichoic acid from *Staph aureus* In polysacchar[illegible]

[illegible] exchange columns and contained 5 amino acids Tanned cell sensitizing antigens seem to be present in most strains of *Staph aureus* and in certain strains of *Staph epidermidis*

An antigen sensitizing normal sheep erythrocytes [illegible]

[illegible] antigen is not present in purified protein A purified polysaccharide A or *Staph aureus* wall teichoic acid It was isolated from extract A on the ion exchange column and contained glucuronic acid and ribose

been described by *Oeding & Haukenes* (20) It may be due to small molecular differences in the protein A antigen which stimulate the formation of two corresponding antibodies As it has not been possible to separate two substances corresponding to the lines, they must have very similar properties The antibody or antibodies in Cowan I antiserum responsible for the double line on agar, may therefore be the complete antibody response to the antigen, whereas the antibody in normal human serum may be incomplete This may be of interest in connection with the rather peculiar fact that all normal human sera contain this antibody

It is also interesting that normal human sera seem to contain antibodies in high titres directed against the antigens in *Staph aureus* sensitizing tanned sheep cells We were not able to demonstrate antibodies in a limited number of normal human sera against the antigen sensitizing normal sheep cells Antibodies against presumably the same substance have, however, been found in normal human sera by others (22, 21, 16)

The antigen in extract A sensitizing normal sheep cells has the properties of the Rantz antigen (21) It was demonstrated in varying amounts in strains of *Staph aureus*, in *Staph epidermidis* and *B subtilis*, but not in *M lysodeikticus* Further it is sensitive to heat at alkaline but not at acid pH The antigen is undoubtedly identical to that described by *Jensen et al* (11) in extract A There does not seem to be more than one substance in *Staph aureus* sensitizing normal erythrocytes, although this possibility has not been systematically investigated It is, therefore, likely that the sensitizing substances described by other authors (22, 19, 5) are identical to that described here

The antigen sensitizing normal sheep cells is not identical to any of the other antigens present in extract A This was shown by absorption of the extract and antiserum, and by fractionation on ion exchange column The antigen seems to be quite loosely attached to the other antigenic substances in extract A, as it could easily be isolated on the column It is therefore not present in the purified protein A or polysaccharide A preparations As it is not destroyed by trypsin and shown to contain glucuronic acid and ribose, the antigen is apparently a sugar *Gorzynski et al* (1) suggest that the Rantz antigen may be related to teichoic acid Our experiments did not support this theory First, the teichoic acid preparations examined and polysaccharide A had not the ability to sensitize normal sheep cells Second, the isolated antigen sensitizing normal cells was not a teichoic acid

The antigens sensitizing tanned sheep cells appear to be widely distributed in *Staph aureus* strains In polysaccharide A the antigen seems to be bound to the mucopeptide moiety contaminating the precipitinogen (polysaccharide A), which is serologically identical to the wall teichoic acid of *Staph aureus* H The teichoic acid preparations examined, had no sensitizing antigen Although the substance in polysaccharide A

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An antigen sensitizing normal sheep erythrocytes was found in washings and crude extracts of *Staph. aureus* strains and in strains of *Staph. epidermidis* and *B. subtilis*, but not in *M. lysodeikticus*. This antigen is apparently of the Rantz type. The antigen is not present in purified protein A, purified polysaccharide A or *Staph. aureus* wall teichoic acid. It was isolated from extract A on the ion exchange column and contained glucuronic acid and ribose.

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The precipitinogens protein A and polysaccharide A and the sensitizing substances were shown on absorption and column fractionation to be different antigens

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In the case about to be described, *Past pseudotuberculosis* X was isolated from man The only previous examples of this are the two cases published by *Hassig* in 1949

CASE HISTORY

A 21 year old man previously well was admitted to the surgical clinic with a history of 24 hours of abdominal pain The pain started in the epigastrium but had migrated down to the right iliac fossa There was nausea but no vomiting Stool was normal No diarrhoea had recently been noted The patient denied contact with animals The temperature on admittance was 37 7° C and the white count 12 600 There was distinct tenderness on palpation over McBurney s point but no muscular defence nor a palpable mass was noted No tenderness was found at rectal examination

q e according t t arker Keer (*Schackelford* 1956) There was no soiling at all One of the inflamed lymph nodes was extirpated and halved One half was sent using sterile precautions for bacteriological and the other for pathological examination

The postoperative course was quite uneventful No antibiotics were administered The patient was discharged from hospital on the third postoperative day The wound healed primarily in a week and he resumed work after four weeks He had since had no abdominal complaints Four and a half months postoperatively, a small bowel series using barium contrast showed a slight narrowing of the distal five cms of the ileum However a repeat examination six month postoperatively was normal

Histo-Pathology

The appendix was swollen and reddened only in the proximal part and had a persisting lumen Histologically, the mucosa was largely preserved, but with abundant lymphoid tissue with big reaction centres In the proximal part it showed patches of oval or irregular necrosis with closely packed polymorphonuclear leucocytes and nuclear débris A few small vessels contained fibrin thrombi In the surroundings there were fairly numerous plasma cells, some reticulocytes and single eosinophilic leucocytes but no giant cells or granulomas No lesions were found in the distal part of the appendix The submucosa was oedematous with streaks of round cells and leucocytes, the inflammatory reaction extending to the serosa, which was partly covered with fibrin

Acta path. et microbiol. scandinav. 62: 128–132, 1964

The Institute of Clinical Bacteriology (Head: Professor S. Winblad), The Department of Surgery (Head: Professor H. Wulff) and The Institute of Pathology (Head: Professor F. Linell), Malmö General Hospital, University of Lund, Sweden.

A CASE OF HUMAN INFECTION WITH PASTEURELLA PSEUDOTUBERCULOSIS X

By

M. G. Carlsson, H. Ryd and N. H. Sternby

Received 9.3.64

Since the end of the last century *Pasteurella pseudotuberculosis* has been known to cause infections in animals, especially rodents. The term pseudotuberculosis was created by the pathologist *C. J. Eberth*, who described the illness in guinea pigs and rabbits in 1885–86. The bacterium was isolated by *Malassez & Vignal* at the same time in 1884.

From the first half of the twentieth century only some twenty human cases have been reported. In 1949 *Hassig* made a summary review of these while presenting two cases of his own. Most of the cases were septic typhoidal conditions and the mortality was high. In 1952 *Pucchaud* described a case resembling appendicitis with mesenteric lymphadenites from which *Pasteurella pseudotuberculosis* was isolated. Among the cases of acute abdomen that *Masshoff* described in 1953 he set apart one abscessforming reticulocytic mesenteric lymphadenopathy in the ileocoecal angle presenting a clinical picture indistinguishable from that of acute appendicitis. Further investigations by *Knapp & Masshoff* showed the condition to be associated with the presence of *Past. pseudotuberculosis* (1954). Other clinical manifestations such as subacute or chronic appendicitis, enteritis and gastroenteritis were described. In some instances it was possible to isolate the organism but more often the diagnosis was established by clinical, serological and histological examinations.

A number of reviews and reports show that the infection is not uncommon in man (*Knapp* 1959, *Mair et al.* 1960, *Arnulf* 1960, *Mollaret* 1960, *Schmidt* 1960, *Beerens* 1960, *Kiaer* 1960, *Daniels* 1961, *Randall & Mair* 1962, *Boettger* 1962, *Texier* 1962).

However, only a small number of non specific infections in the ileocoecal region can be referred to *Past. pseudotuberculosis*. In most cases the aetiology remains unknown.

In 1962 *Daniels* described a bacterium isolated from chincillas and resembling *Past. pseudotuberculosis*. Its morphological and cultural properties were compatible with *Past. pseudotuberculosis* but it differed from the previously known groups I–V with regard to biochemistry

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Acute appendicitis being suspected an acute lapar tomy was done The appendix was found free and mobile 2 3 cms of its base was inflamed swollen and firm whereas its distal part was thin and pale and without signs of inflammation There was minimal extension of the inflammation to the adjacent coecum which was otherwise normal The terminal 10 cms of the ileum was reddened thickened

[illegible]

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12th 1/160 May 28th 1/20 It was thus not possible to demonstrate an O antigen common to the strain of the patient and to *Past pseudotuberculosis I–V* In the course of his illness however the patient formed immune bodies against the O antigen of his own strain

Using the term strain 897 Winblad *Knapp & Thal* (1963) studied the relations between the pasteurella strain of the patient and the strain isolated from chinchillas (*Daniels* 1962) They found that the two strains isolated from human material were identical and that they had the cultural biochemical properties characteristic of those strains that had been isolated from animals Pasteurella phages did not attack any pasteurella X strain Further details of these matters and the differential diagnosis between *Past pseudotuberculosis I V* and pasteurella X are given in the article of *Knapp & Thal*

SUMMARY

In one case of acute terminal ileitis in man spreading to the appendix and mesenteric lymph nodes a bacterium resembling *Pasteurella pseudotuberculosis* could be demonstrated It proved identical (*Knapp & Thal*) with a strain isolated in 1949 (*Hassig*) from 2 patients suffering from septical typhoidal lethal conditions and it had the same cultural biochemical properties as pasteurella X strain isolated from animals The histopathological picture of a mesenteric lymph node was non specific whereas changes occurring in the lymphoid tissue of the appendix showed some similarity to those described by *Masshoff* (1953) The clinical symptoms corresponded to an acute appendicitis and no complications occurred In the patient s serum there was a significantly rising and then subsiding titre of O agglutinins against the bacterium which was found to have no O antigen in common with *Pasteurella pseudotuberculosis* strains I V

ADDENDUM

After this article had been finished one more case with positive serology was observed A man 39 years old was taken ill on Nov 14th with symptoms indicating acute appendicitis On appendectomy Nov 15th the appendix was found to be normal whereas the terminal part of the ileum was thickened and of a strong red colour Only small mesenteric lymph nodes were seen Histological examination was not done On Nov 22nd O antigen of *Past pseudotuberculosis* V was agglutinated by the patient s serum in dilution 1 160 ...

The lymph node was the size of a bean, with homogenous grey cut surface Histologically the structure was well-preserved The sinuses were often widened with swollen endothelial cells and contained plasma cells and leucocytes There were no proliferating reticular cells, granulomas, giant cells, or necrosis

The pathological-anatomical examination thus showed no specific lymphoglandular changes, but in the proximal part of the mucosa of the appendix there were patchy necroses These changes differ from those described as typical of acute mesenteric lymphadenitis (*Vasshoff*) In the letter, round or starshaped necroses crammed with leuco cytes are usually seen, surrounded by a zone of reticular cells, and also uncharacteristic giant cells are sometimes seen The changes occur mainly in lymph nodes and only seldom in the mucosa of the appendix In our case, conditions were reversed

Bacteriology

Material from a mesenteric lymph node was enriched with peptone and glucose in ordinary placentar broth at 37° C for 48 hours Blood agar plates were inoculated and incubated aerobically both at 37° C and 22° C As early as 48 hours later, there was a rich growth of yellow-grey, matt colonies, partly with a smooth and partly with a nodular surface and sharp edge In Gram stained smears from the colonies, small slender, sometimes polestained Gram negative rods were seen, showing a moderate degree of pleomorphism in older colonies On cultivation in U-tubes with semi-solid agar, the bacterium was motile at 22° C, but non-motile at 37° C Its cultural and morphological characteristics were consequently those of *Pasteurella pseudotuberculosis*

The bacterium rapidly produced acid but no gas with mannitol, saccharose, dextrose, and levulose, and more slowly with zylose, maltose, arabinose, and galactose The indole, H_2S, and oxidase reactions were negative, the nitrate and catalase reactions positive Urease was produced on LSU agar (*Juhlin & Ericson* 1961) Lactose, inositol, rhamnose, raffinose, inulin, aesculin, and salicin were not influenced In producing acid with saccharose, but not with rhamnose of aesculin, the strain differed from *Past pseudotuberculosis In vitro*, using disc technique, the strain proved insusceptible to sulphonamides, penicillin, and erytromycin slightly sensitive to kanamycin, and sensitive to streptomycin, tetracyclines and chloramphenicol

Serology

O antisera from rabbits immunized with *Past pseudotuberculosis I–V* did not agglutinate O antigen prepared from the strain of this patient The patient's serum did not agglutinate O antigen belonging to pasteurella strains I–III The patient's own O antigen, however, was agglutinated by his serum, showing the following titres Jan 15th 0, Febr

12th 1:160 May 28th 1:20 It was thus not possible to demonstrate an O antigen common to the strain of the patient and to *Past pseudotuberculosis I–V*. *In* the course of his illness however the patient formed immune bodies against the O antigen of his own strain.

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Institute of Virology University of Uppsala Sweden

ROLE OF INTERFERON IN THE AUTOINTERFERENCE OF NEWCASTLE DISEASE VIRUS (NDV)

By

SVANTE HERMODSSON

Received 27 ii 64

In titrations especially of myxo and arboviruses it is a common observation that higher concentrations of virus induce less cellular damage and formation of less virus than lower virus concentrations This phenomenon has been called autointerference, zone phenomenon or autoinhibition and has been ascribed to the presence of interferon or incomplete virus in the virus suspensions (for references see 14)

In a previous communication (12) it was shown that the autointerference of NDV in calf cultures can be suppressed by a concurrent infection with parainfluenza virus type 3 This effect appeared to be due to suppression of an interferon like inhibitor The present study was undertaken in order to characterize the inhibitor and to establish its role in the autointerference of NDV in calf kidney cultures

MATERIALS AND METHODS

Cell cultures Primary c ll res f alf k dn
kidney tissue of newb
roller tubes or plastic
with 0 5 per cent lact
(100 I U penicill n a

The k

nunization of rabbits with
The serum had a haemag
—20 C o 1ologous virus and was stored at

Virus titration Infectivity was as a rule determined in roller tube cultures of calf kidney cells Each of 5 tubes was inoculated with 0 1 ml of serial tenfold dilutions of the virus samples The cultures were examined for cytopathic changes and haem adsorbing capacity after incubation for 5 to 7 days The haemadsorption test was carried out according to the modification of *Chanock et al* (3) The number of TCD_{50} was calculated by Kärber s method

Plaque assay of NDV was performed on plastic petri dishes with confluent sheets of HeLa cells After washing with phosphate buffered saline (PBS) the plates were inoculated with 0 1 ml of tenfold dilutions of the samples After an adsorption period of 30 minutes at 37° C the plates were overlaid with 5 ml MEM containing 10 per cent horse serum and 0 95 per cent agar (Special agar Noble Difco) The plates were stained after incubation for 2 days with 2 ml agar overlay containing neutral red in a final concentration of 1/20 000 The plaques were counted on the 4th day of incubation

Plaque assay of bovine enterovirus was performed similarly on calf kidney cells grown in prescription bottles The overlay for these cells was Earle s salt solution with 2 2 g $NaHCO_3$ per litre 0 5 per cent lactalbumin hydrolysate 2 per cent calf serum and 0 95 per cent agar

HA titrations were performed in tubes by the pattern test Serial twofold dilutions of the virus material in 0 15 M NaCl were mixed with an equal volume (0 5 ml) of a 0 5 per cent suspension of guinea pig erythrocytes The tests were read after 2 hours at 4° C and the titre was expressed as the reciprocal of the highest initial dilution giving partial agglutination

Assay for interferon activity Prior to titration the samples were centrifuged at 80 000 g for 2 hours Serial twofold dilutions of the supernatants were then made in maintenance medium containing NDV antiserum at a final dilution of 1/80 The dilutions were incubated at 37° C for 2 hours and inoculated into roller tubes Each dilution was inoculated into 2 tubes and each tube received 1 ml medium After incubation for 18 to 24 hours the cultures were exposed to a challenge infection with 10^4 TCD_{50} of Sendai virus The cultures were incubated for another 3 to 4 days and were subsequently examined for cytopathic changes and haemagglutinating activity The interferon titre was expressed as the reciprocal of the highest dilution giving at least a fourfold reduction of the HA titre compared with the controls

Staining with fluorescent antibodies Subcultures of calf kidney cells grown on coverslips in Leighton tubes were washed in PBS fixed in aceton and air dried Staining with fluorescent antibodies was then performed by the indirect method (25) with NDV antiserum diluted 1 10 in PBS and with goat antirabbit globulin conjugated with fluorescein isothiocyanate (Microbiological Associates Bethesda Maryland) The coverslips were mounted on object glasses in glycerol phosphate buffer and examined in a Zeiss fluorescence microscope equipped with a darkfield condenser and a mercury lamp (Osram HBO 200) The primary filter was BG 12 and the secondary Zeiss 50 alone or in combination with Zeiss 47

Other procedures In order to inactivate interferon the virus suspensions were treated at 37° C for 2 hours with 0 5 mg of crystallized trypsin (Worthington Biochem Corp New Jersey) per ml The enzyme was inactivated by addition of 1 mg of soybean trypsin inhibitor (Mann Research Lab New York) per ml Treatments with DNase and RNase (Worthington Biochem Corp New Jersey) were performed at 37° C for 30 minutes with the enzymes in concentrations of 100 µg per ml

The ether sensitivity of the inhibitor was tested by shaking the inhibitory fluid with 20 per cent ether for 30 minutes at 4° C The ether was removed by evaporating overnight at 4 C and then bubbling nitrogen gas through

0 07 M HCl KCl buffer
d to pH 7 4 by further
maintenance medium

for 12 hours

RESULTS

Demonstration of Autointerference

An inhibited virus multiplication was regularly observed in calf kidney cultures inoculated with undiluted suspensions of NDV Fig 1

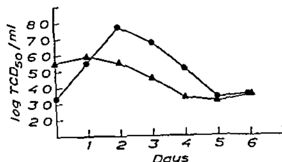


Fig 1
Growth curves of NDV in flask cultures of calf kidney cells, ▲——▲ inoculated with undiluted virus suspension ●——● virus diluted 10^{-2}

shows the virus yields in flask cultures inoculated either with 1 ml of the undiluted stock suspension or with 1 ml of the stock diluted 10^{-2}, corresponding to input *multiplicities of approximately 1 and 0 01* respectively The medium was changed daily and assayed for infectivity and haemagglutinin Before titration one part of the harvest was subjected to ultrasonic vibration (11) with the idea that NDV like several other paramyxoviruses (11, 21) produces haemagglutinin which can be unmasked by this treatment

TABLE 1
Production of Haemagglutinin in Calf Kidney Cultures Inoculated with Different Amounts of NDV

Dilution of NDV inoculated	HA units ml culture fluid at indicated days after inoculation of NDV					
	1	2	3	4	5	6
10^{0}	<2 (2)*	2 (4)	<2 (<2)	<2 (<2)	<2 (<2)	<2 (<2)
10^{-2}	<2 (<2)	32 (64)	32 (64)	<2 (<2)	<2 (<2)	<2 (<2)

* Figures in brackets refer to titres after ultrasonic treatment

As shown in Fig 1 and Table 1 the production of infectious virus and haemagglutinin was significantly suppressed in the cultures inoculated with undiluted virus suspensions Such cultures continuously produced low titres of infectious virus for a long period (Fig 2) A persistence of the NDV infection also occurred in secondary cultures prepared by trypsination of primary cultures on day 2 after virus inoculation (Fig 2)

The content of viral antigen in the cells was studied by the fluorescent antibody technique Monolayers of calf kidney cells grown on coverslips were infected with NDV at input multiplicities of approximately 5 and 0 05 respectively At intervals after inoculation the cells were fixed in acetone and the media assayed for haemagglutinating

Virus titration. Infectivity was as a rule determined in roller tube cultures of calf kidney cells. Each of 5 tubes was inoculated with 0.1 ml of serial tenfold dilutions of the virus samples. The cultures were examined for cytopathic changes and haemadsorbing capacity after incubation for 5 to 7 days. The haemadsorption test was carried out according to the modification of *Chanock et al.* (3). The number of TCD_{50} was calculated by Karber's method.

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Plaque assay of bovine enterovirus was performed similarly on calf kidney cells grown in prescription bottles. The overlay for these cells was Earle's salt solution with 2.2 g $NaHCO_3$ per litre, 0.5 per cent lactalbumin hydrolysate, 2 per cent calf serum and 0.95 per cent agar.

HA titrations were performed in tubes by the pattern test. Serial twofold dilutions of the virus material in 0.15 M NaCl were mixed with an equal volume (0.5 ml) of a 0.5 per cent suspension of guinea pig erythrocytes. The tests were read after 2 hours at 4° C and the titre was expressed as the reciprocal of the highest initial dilution giving partial agglutination.

Assay for interferon activity. Prior to titration the samples were centrifuged at 80 000 g for 2 hours. Serial twofold dilutions of the supernatants were then made in maintenance medium containing NDV antiserum at a final dilution of 1/80. The dilutions were incubated at 37° C for 2 hours and inoculated into roller tubes. Each dilution was inoculated into 2 tubes and each tube received 1 ml medium. After incubation for 18 to 24 hours the cultures were exposed to a challenge infection with 10^4 TCD_{50} of Sendai virus. The cultures were incubated for another 3 to 4 days and were subsequently examined for cytopathic changes and haemagglutinating activity. The interferon titre was expressed as the reciprocal of the highest dilution giving at least a fourfold reduction of the HA titre compared with the controls.

Staining with fluorescent antibodies. Subcultures of calf kidney cells grown on coverslips in Leighton tubes were washed in PBS, fixed in aceton and air dried. Staining with fluorescent antibodies was then performed by the indirect method (25) with NDV antiserum diluted 1 : 16 in PBS and with goat antirabbit globulin conjugated with fluorescein isothiocyanate (Microbiological Associates, Bethesda, Maryland). The coverslips were mounted on object glasses in glycerol phosphate buffer and examined in a Zeiss fluorescence microscope equipped with a darkfield condenser and a mercury lamp (Osram HBO 200). The primary filter was BG 12 and the secondary Zeiss 50 alone or in combination with Zeiss 47.

Other procedures. In order to inactivate interferon the virus suspensions were treated at 37° C for 2 hours with 0.5 mg of crystallized trypsin (Worthington Biochem. Corp., New Jersey) per ml. The enzyme was inactivated by addition of 1 mg of soybean trypsin inhibitor (Mann Research Lab., New York) per ml. Treatments with DNase and RNase (Worthington Biochem. Corp., New Jersey) were performed at 37° C for 30 minutes with the enzymes in concentrations of 100 μg per ml.

The ether sensitivity of the inhibitor was tested by shaking the inhibitory fluid with 20 per cent ether for 30 minutes at 4° C. The ether was removed by evaporating overnight at 4° C and then bubbling nitrogen gas through.

Dialysis at pH 2.0 was performed in cellulose casings against 0.07 M HCl-KCl buffer for 18 hours at 4° C. The pH of the dialysed fluid was restored to pH 7.4 by further dialysis against Earle's salt solution for 12 hours and against maintenance medium for 12 hours.

RESULTS

Demonstration of Autointerference

An inhibited virus multiplication was regularly observed in calf kidney cultures inoculated with undiluted suspensions of NDV. Fig. 1

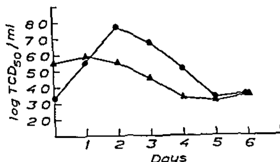


Fig 1

Growth curves of NDV in flask cultures of calf kidney cells ▲———▲ inoculated with undiluted virus suspension ●———● virus diluted 10^{-2}

shows the virus yields in flask cultures inoculated either with 1 ml of the undiluted stock suspension or with 1 ml of the stock diluted 10^{-2}, *corresponding to input multiplicities of approximately 1 and 0 01 respectively* The medium was changed daily and assayed for infectivity and haemagglutinin Before titration one part of the harvest was subjected to ultrasonic vibration (11) with the idea that NDV like several other paramyxoviruses (11, 21) produces haemagglutinin which can be unmasked by this treatment

TABLE 1

Production of Haemagglutinin in Calf Kidney Cultures Inoculated with Different Amounts of NDV

Dilution of NDV inoculated	HA units/ml culture fluid at indicated days after inoculation of NDV					
	1	2	3	4	5	6
10^{0}	<2 (2)*	2 (4)	<2 (<2)	<2 (<2)	<2 (<2)	<2 (<2)
10^{-2}	<2 (<2)	32 (64)	32 (64)	<2 (<2)	<2 (<2)	<2 (<2)

Figures in brackets refer to titres after ultrasonic treatment

As shown in Fig 1 and Table 1 the production of infectious virus and haemagglutinin was significantly suppressed in the cultures inoculated with undiluted virus suspensions Such cultures continuously produced low titres of infectious virus for a long period (Fig 2) A persistence of the NDV infection also occurred in secondary cultures prepared by trypsination of primary cultures on day 2 after virus inoculation (Fig 2)

The content of viral antigen in the cells was studied by the fluorescent antibody technique Monolayers of calf kidney cells grown on coverslips were infected with NDV at input multiplicities of approximately 5 and 0 05 respectively At intervals after inoculation the cells were fixed in acetone and the media assayed for haemagglutinating

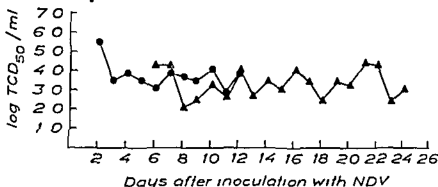


Fig 2
Yield of infectious virus in persistently infected cultures, ●——● primary cultures, ▲——▲ secondary culture, subcultivated 2 days after inoculation of undiluted virus suspension

activity In cultures exposed to the higher multiplicity of infection the majority of cells contained fluorescent material one day after inoculation The intensity of the fluorescence varied and in many cells there were only a few fluorescent granules (Fig 3 A) The fluorescence disappeared after further incubation (Fig 3 B) and 4 days after inoculation fluorescence could be demonstrated in only about one per cent of the cells (Fig 3 C) Fluorescence was more intense in cultures inoculated with NDV at a multiplicity of about 0 05 One day after inoculation the fluorescence occurred in only small scattered foci of cells, and on the second day almost all cells showed bright fluorescence (Fig 3 D) Further incubation of these cultures resulted in a marked cellular degeneration which was not observed in cultures inoculated with NDV at the higher multiplicity

Participation of Interferon in the Autointerference

To examine whether the interfering activity of the virus stocks could be separated from the infectivity, samples of the stock suspensions were centrifuged at 55,000 g for 90 minutes The supernatant was collected and the pellet resuspended in a volume of maintenance medium corresponding to the original volume The stock suspension was further treated with trypsin since this enzyme inactivates interferon (17) but not NDV (8) The multiplication of NDV was then followed in flask cultures inoculated with the untreated virus stock, the resuspended pellet, the trypsin treated virus or with both the supernatant and the trypsin-treated suspension Each culture was inoculated with 1 ml of the different preparations

As shown in Table 2 the multiplication of NDV was inhibited in cultures inoculated with untreated virus or with a mixture of trypsin-treated virus and supernatant Higher titres of virus were produced in cultures inoculated with only the trypsin-treated virus or the resuspended pellet These results indicated that the autointerference pheno



Fig 3

Micrographs of calf kidney cells infected with NDV and stained with fluorescent antibodies against this virus. Magnification × 600. A–C show cultures inoculated with NDV at a multiplicity of about 5 and incubated for 1, 2 and 4 days respectively. D shows a culture inoculated with NDV at a multiplicity of about 0.05 and incubated for 2 days.

menon observed in cultures inoculated with undiluted virus suspensions was mainly due to the presence of an inhibitor similar to interferon The inhibitor was therefore examined for some of the properties which characterize interferon (17, 20)

TABLE 2
Inhibition of NDV Multiplication by a Non-Viral Factor in the Virus Suspensions

Inoculated suspension	Cell degeneration per cent	Activity per ml culture fluid	
		Log TCD$_{50}$	HA units
Untreated virus	No	5 4	<4
Resuspended sediment*	About 50	7 4	32
Trypsin treated virus	About 50	7 6	32
Trypsin treated virus + Supernatant*	No	5 5	<4

* After centrifugation at 55 000 g for 90 minutes

Like interferon (23) the NDV inhibitor was precipitated by ammonium sulphate A high-titered preparation was obtained by the addition of 500 g of ammonium sulphate to 1000 ml of infectious culture fluid The sediment obtained after 18 hours at 4° C and centrifugation at 1,500 g for 30 minutes was resolved in 20 ml of medium and dialysed at 4° C against Earle's salt solution for 1 day and against maintenance medium for 1 day Virus was removed by 2 centrifugations at 80,000 g for 2 hours The supernatant obtained after the last centrifugation was diluted in PBS and treated with ether, trypsin, acid, DNase or RNase as described in Materials and Methods The heat resistance of the inhibitor was tested by incubation at 70° C for 30 minutes and the sedimentation after centrifugation at 100,000 g for 2 hours The inhibitor was further treated with NDV-antiserum for 2 hours at 37° C The antiviral activity of the inhibitor was determined before and after the different treatments according to the technique described in Materials and Methods for assay of interferon activity

TABLE 3
Properties of the NDV Inhibitor

Treatment	Inhibitor titre	
	Before treatment	After treatment
Centrifugation at 100 000 g for 2 hours	4	4
Incubation with NDV antiserum	4	4
Dialysis against acid pH 2 0	4	4
Incubation at 70° for 30 minutes	8	8
Shaking with ether	16	8
Incubation with trypsin	16	<2
Incubation with RNase	8	8
Incubation with DNase	8	8

As shown in Table 3 dialysis at pH 2 0, incubation at 70° C, as well as treatment with nucleases or NDV-antiserum did not inactivate the inhibitor Nor was the inhibitor sedimented by centrifugation at 100,000 g for 2 hours Treatment with trypsin, on the other hand, clearly reduced the antiviral activity and a minor reduction in titre was obtained after shaking with ether The inhibitor thus appeared to have several properties in common with interferon

The Production of *Interferon*

It was further studied whether interferon was produced in cell cultures where the virus multiplication was inhibited by autointerference The experiment was performed in flask cultures infected with NDV at an input multiplicity of about 1 and 0 01 respectively The medium was changed daily and assayed for interferon activity Interferon in the cell phase was determined after disintegration of the cells by ultrasonic treatment for 5 minutes (11) Prior to this treatment the cells were washed three times in maintenance medium, loosened from the glass wall with a platinum wire loop and suspended in 10 ml of medium

As shown in Table 4 interferon was produced in cultures inoculated with undiluted virus suspensions, but the maximum titre was about 4 times lower in these cultures than in the cultures inoculated with 100 fold less virus No interferon could be demonstrated after 3 days of incubation in the former cultures It was also observed that the release of interferon appeared to be somewhat delayed in relation to virus multiplication (for comparison see Fig 1)

TABLE 4
Production of Interferon in NDV Infected Cultures

Days after inoculation of NDV	Interferon titre			
	Medium		Disintegrated cells	
	Dilution of NDV		Dilution of NDV	
	10^{0}	10	10^{0}	10^{-2}
1	1	<1	4	<2
2	4	4	1	16
3	2	16	<1	16
4	<1	2	<1	ND
5	<1	<1	<1	ND

ND = not done

Resistance against Challenge Infection

The antiviral resistance of NDV-infected and interferon ... tures ...
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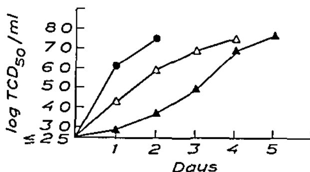


Fig 4

Resistance of calf kidney cultures to challenge infection with a bovine enterovirus after a previous infection with NDV (▲——▲) or treatment with NDV interferon (△——△) Control inoculated with challenge virus only (●——●)

a final interferon titre of 16 in the culture fluid NDV-antiserum had previously been added to the interferon preparation in order to neutralize remaining viral activity After incubation for 3 days at 37° C these two sets of cultures and control cell cultures were inoculated with 10^{3} TCD_{50} of the enterovirus strain 80/61 The multiplication of the challenge virus was then followed by assaying the media for infectivity at intervals after infection

The reproduction of the challenge virus was found to be inhibited both in cultures infected with NDV and in cultures treated with interferon (Fig 4) The inhibition of the virus multiplication was, however, greater in the NDV infected cultures, although less interferon could be demonstrated in these cultures than in cultures, supplied with exogenous interferon Similar results were obtained when pseudorabies virus parainfluenza virus type 1 or foot and-mouth disease virus were used for challenge

Adsorption of Virus to the Cells

In several investigations dealing with viral interference the adsorption of challenge virus to the cells has not been found to be impaired (2, 10, 16, 22, 24, 26) However, a reduced ability of the cells to adsorb superinfecting virus has been established in the homologous interference between UV irradiated and active NDV in chick cells (1) Adsorption of NDV to cells showing autointerference was therefore examined Since NDV infected cultures were more resistant against bovine enterovirus than interferon-treated cells the adsorption of the latter virus was also examined

The experiments were performed with calf kidney cells grown in plastic petri dishes inoculated with 0 2 ml of the NDV stock suspension and incubated for 4 days at 37° C prior to the addition of the superinfecting virus Other cultures were inoculated with 0 4 ml of NDV interferon and incubated for 2 days, some additional cultures were left uninoculated The plates were then washed 4 times with mainte-

nance medium and inoculated with either 10^6 TCD_{50} of NDV or 10^5 TCD_{50} of enterovirus 80 61 in volumes of 0 2 ml Virus was allowed to adsorb at 37° C and the plates were shaken regularly in order to spread the inoculum as a thin film on the cell sheet After different periods of adsorption 2 ml of maintenance medium was added to the plates and the remaining viral activity of this fluid was assayed by plaque titration The reduction of the titres was considered to be an estimate of the ability of the cells to adsorb virus

After an adsorption period of 30 minutes approximately 10 per cent of the added amount of enterovirus was recovered in the supernates of *control cultures and interferon treated cultures* On the other hand, in cultures previously infected with NDV 60 per cent of the infectivity was still unadsorbed Similar differences were obtained at an adsorption period of 60 minutes and when the cultures were challenged with NDV (Table 5)

TABLE 5

Adsorption of Virus to the Cells in Cultures Previously Infected with NDV or Treated with NDV Interferon

Challenge virus	Adsorption in minutes	Per cent residual infectivity in the fluid phase		
		NDV infected	Interferon treated	Untreated
NDV	30	77	28	31
NDV	60	90	14	25
Enterovirus	30	60	14	10
Enterovirus	60	68	18	14

DISCUSSION

The experiments reported here show that primary cultures of calf kidney cells infected with NDV produce a substance with [illegible] inhibitory [illegible] respects [illegible] al (17) [illegible] considered to be an interferon substance Production of interferon by infection with NDV has previously been shown in MCN cells (9), mouse embryo cells (18), and in chick embryos (19).

The autointerference phenomenon which appeared in calf [illegible] [illegible] was suppressed in cultures inoculated with virus suspensions from which interferon had been removed by dilution, ultracentrifugation or treatment with trypsin

The presence of interferon in the inocula did not prevent synthesis of viral protein in the NDV-infected cultures After inoculation of undiluted virus suspensions and staining with fluorescent antibodies the majority of cells contained viral antigen one day after inoculation The

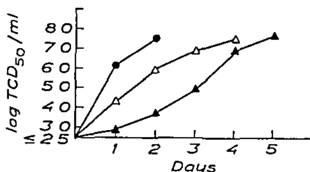


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DISCUSSION

The experiments reported here show that primary cultures of calf kidney cells infected with NDV produce a substance with an inhibitory effect on virus multiplication The inhibitor appears in several respects to be similar to the interferon originally described by *Isaacs et al* (17) and is therefore considered to be an interferon substance Production of interferon by infection with NDV has previously been shown in MCN cells (9), mouse embryo cells (18), and in chick embryos (19)

The autointerference phenomenon which appeared in calf kidney cultures inoculated with undiluted suspensions of NDV *seemed to be due* to the presence of interferon in the inoculum This was supported by the fact that the autointerference was suppressed in cultures inoculated with virus suspensions from which interferon had been removed by dilution ultracentr[illegible]

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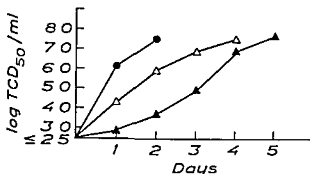


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production of interferon but also by a reduced ability of the cells to adsorb virus

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amount of fluorescent material, however, was significantly less in these cultures than in the cultures inoculated with virus at low multiplicities. The immunofluorescence also disappeared rapidly after further incubation of the cultures inoculated with undiluted virus suspensions without any obvious degeneration of the cells. These cultures continuously produced low titres of infectious virus even after subcultivation of the cells and thus appeared to be persistently infected with NDV. A persistent infection of calf cells with NDV has previously been observed by *Dannacher & Fedida* (6).

It has further been investigated whether virus multiplication in the persistently infected cultures is inhibited by interferon. Interferon was only detected during the first 3 days after inoculation of NDV although the titres were lower in these cultures than in cultures with more active virus multiplication. Synthesis of interferon was not demonstrated at a later stage of the persistent infection unless interferon was assayed by a more sensitive method (13) than that used in this report. On the basis of these results and in agreement with other investigations (4, 7, 9, 15, 22) it can be assumed that interferon plays a rôle for the establishment and maintenance of antiviral resistance in persistently infected cultures.

With regard to the fact that only small amounts of interferon have been demonstrated in the persistently infected cultures after the initial stage of infection (13), it must be questioned whether interferon is the only factor which maintains the carrier state. Interference in the absence of interferon has also been described. In HeLa cells chronically infected with Coxsackie virus *Crowell & Syverton* (5) found no interferon but the adsorption and penetration of the virus was decreased. However, in some other systems of persistent infections (2, 22) interferon has been demonstrated, but the adsorption of virus to the cells was not impaired. The results of the present investigation indicate that the virus adsorption is inhibited in persistently infected cultures but not in interferon-treated cultures.

The antiviral resistance was also greater in persistently infected cultures than in interferon-treated. This difference might be explained by the impaired virus adsorption in the persistently infected cultures, but it is still possible that the greater antiviral resistance is mainly due to a stronger action of endogenous interferon.

SUMMARY

The autointerference phenomenon which appears in calf kidney cultures infected with NDV has been associated with the presence of interferon in the inoculum. This amount of interferon does not prevent synthesis of viral protein but significantly reduces the virus yields and the cytopathic effect. The inoculation of undiluted NDV suspensions induces a persistent infection which is probably maintained not only by

production of interferon but also by a reduced ability of the cells to adsorb virus

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Table 1 second part

Time after hepatectomy	Control	Irradiation	Irradiation and hepatectomy	Hepatectomy
2 days	38.3 ± 2.7	39.5 ± 2.2	26.7 ± 2.8	26.2 ± 2.5
14 days	41.7 ± 4.8	42.4 ± 5.9	42.2 ± 4.7	38.6 ± 6.9

Should be

Time after hepatectomy	Control	Irradiation	Irradiation and hepatectomy	Hepatectomy
2 days	38.3 ± 2.7	43.5 ± 2.5	30.1 ± 2.7	26.2 ± 2.5
14 days	41.7 ± 4.82	40.3 ± 4.8	41.5 ± 4.7	38.6 ± 6.8

Acta path. et microbiol. scandinav. 62: 145–150, 1964

The Department of Thoracic Surgery, (Heads: Jens I. Hansen, M.D. and Thomas Castberg, M.D.) and The Department of Pathology (Head: Charles Johansen, M.D.), Bispebjerg Hospital, Copenhagen, Denmark.

THE INCIDENCE OF LOCAL RECURRENCE AND DISTANT METASTASES IN SURGICALLY TREATED CASES OF LUNG CANCER

By

POUL S. RASMUSSEN

Received 13.ii.64

The demonstration of circulating tumour cells at the time of operation in patients with cancer of the lung has been a contributory cause of the present frequent use of chemotherapy as an adjuvans to surgical intervention. It is presumed that these tumour cells are particularly vulnerable to the action of cytostatics and it might bee expected that these drugs given at the time of operation will prevent later development of hematogenous metastases in patients subjected to radical operation.

The frequency of circulating tumour cells in patients with cancer of the lung is reported to range from 34 per cent to 80 per cent (*Klassen, Selbach & Sakurai* 1962; *Moore, Sandberg & Watne* 1959; *Pruitt, Hilberg, Morehead & Mengoli* 1962), which is higher than in most other forms of cancer. However, there is no conclusive evidence of the extend to which these cells establish themselves as metastases. *Engell* (1959) found that patients in whom he demonstrated circulating tumour cells at the time of operation lived just as long as those in whom no tumour cells could be demonstrated. According to *Moore et al.* (1959) 95 per cent–99 per cent of the cells perish in the organism.

This study was undertaken to investigate the relative incidence of local recurrence and distant metastases in a group of patients undergoing pulmonary resection for bronchogenic carcinoma. It has been of particular interest to find out how often radically operated patients die from distant metastases without local recurrence, since such metastases might be considered to originate from tumour cells spread to the blood stream imediately prior to and during operation.

I am gratefull to *Johannes Clemmesen, M.D.* for permission to use the files of the *Danish Cancer Registry*.

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The Department of Thoracic Surgery (Heads: Jens I Hansen M D and Thomas Castberg M D) and The Department of Pathology (Head: Charles Johansen M D), Bispebjerg Hospital, Copenhagen Denmark)

THE INCIDENCE OF LOCAL RECURRENCE AND DISTANT METASTASES IN SURGICALLY TREATED CASES OF LUNG CANCER

By

POUL S RASMUSSEN

Received 13 ii 64

The demonstration of circulating tumour cells at the time of operation in patients with cancer of the lung has been a contributory cause of the present frequent use of chemotherapy as an adjuvans to surgical intervention It is presumed that these tumour cells are particularly vulnerable to the action of cytostatics and it might bee expected that these drugs given at the time of operation will prevent later development of hematogenous metastases in patients subjected to radical operation

The frequency of circulating tumour cells in patients with cancer of the lung is reported to range from 34 per cent to 80 per cent (*Klassen, Selbach & Sakurai* 1962 *Moore, Sandberg & Watne* 1959 *Pruitt, Hilberg, Morehead & Mengoli* 1962), which is higher than in most other forms of cancer However, there is no conclusive evidence of the extend to which these cells establish themselves as metastases *Engell* (1959) found that patients in whom he demonstrated circulating tumour cells at the time of operation lived just as long as those in whom no tumour cells could be demonstrated According to *Moore et al* (1959) 95 per cent 99 per cent of the cells perish in the organism

This study was undertaken to investigate the relative incidence of local recurrence and distant metastases in a group of patients undergoing pulmonary resection for bronchogenic carcinoma It has been of particular interest to find out how often radically operated patients die from distant metastases without local recurrence, since such metastases might be considered to originate from tumour cells spread to the blood stream imediately prior to and during operation

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I am gratefull to *Johannes Clemmesen M D* for permission to use the files of the *Danish Cancer Registry*

MATERIAL

The material comprises a consecutive series of 227 patients who all were subjected to lung resection for bronchogenic carcinoma. In another publication (*Rasmussen* 1964) an account will be given of age distribution, types of resection, operative mortality, and survival rate in relation to histology and macroscopic extend of the tumour. In connection with the present study it should be mentioned that very few patients received chemotherapy, and only in cases of advanced recurrence. Apart from an isolated case, x-ray treatment was only given after palliative resections and advanced recurrence.

RESULTS

Operative Findings

The histological classification appears from Table 1. A macroscopical classification ad modum Salzer (1951) has been made with a few minor modifications. In accordance with Salzer the material has been arranged in various main groups, based on the local extend of the tumour, group A comprising cases where the tumour is localized to the lung parenchyma per se, Group B cases with tumour invasion of the visceral pleura, and Group C cases with invasion of the parietal pleura. Included in the latter group are all cases where tumour tissue had to be left in the bronchial stump. In addition, a division has been made based on the degree of lymphogenous spread, grade 1 comprising cases with no demonstrable glandular metastases, grade 2 cases with infiltration of interlobar and hilar glands, and grade 3 cases with infiltration of mediastinal glands. The material classified in this way appears from Table 2.

TABLE 1
Histological Classification

Squamous cell carcinoma parakeratotic	139 cases –	61 per cent
Squamous cell carcinoma polymorphocellular	45 cases	20 per cent
Undifferentiated carcinoma	23 cases	10 per cent
Adenocarcinoma	20 cases	9 per cent
Total	227 cases	100 per cent

TABLE 2
Operative Findings in 227 Cases of Lung Cancer

	Lymphatic involvement			Total
	Grade 1	*Grade 2*	*Grade 3*	
Group A	90	40	30	160
Group B	8	6	6	20
Group C	17	9	21	47
Total	115	55	57	227

The figures read from above downwards represent increasing local extension of the tumour. Group A: tumour limited to the pulmonary parenchyma. Group B: invasion of the visceral pleura. Group C: invasion of the parietal pleura. The figures read from left to right represent increasing lymphatic involvement. Grade 1: no lympnode involvement. Grade 2: involvement of the hilar nodes. Grade 3: involvement of the mediastinal nodes.

Autopsy Findings

The observation period was five to eight years after which 181 patients had died Autopsy was performed in 133 patients

Among 62 patients who died from post operative complications within the first two months after operation, autopsy results are available in 56 cases (90 per cent) Seven of these (14 per cent) had distant metastases which were unrecognized at the time of operation

Among 119 patients *in whom death occurred later than two months* after operation, autopsy results are available in 77 cases (65 per cent) In 13 of these there was no trace of the lung cancer, but in 64 there was local recurrence of the tumour and or distant metastases (See Table 3) Only *ten* patients (16 per cent of those dying from cancer of the lung) had distant metastases without local recurrence Thus, recurrences were a dominant feature being found in 84 per cent of patients dying from cancer In cases where local recurrence and distant metastases appeared simultaneously, the recurrence was often considerable and was usually found to be the primary cause of death

TABLE 3

Autopsy Findings in 64 Patients Who Died Later than Two Months after Pulmonary Resection for Lungcancer

Local recurrence alone	20 cases – 31 per cent
Local recurrence and distant metastases	34 cases – 53 per cent
Distant metastases alone	10 cases – 16 per cent
Total	64 cases – 100 per cent

TABLE 4

Necropsy Findings in Relation to the Local Extension and Lymphatic Spread of Tumour at Time of Operation

	Dead of lung cancer	Recurr alone	Recurr + metast	Total recurr	Metast alone	Recurr metast
Group A	39	10	21	31	8	4/1
Group B	10	5	4	9	1	9/1
Group C	15	5	9	14	1	14/1
Group A_1	20	3	12	15	5	3/1
Group $A_2 + A_3$	19	7	9	16	3	5/1
Group $A_3 + B_3 + C_3$	23	4	16	20	3	7/1

For detailed explanation of the classification see Table 2 and text [illegible]
present cases in which tumour [illegible]
strable lymphnode involvement [illegible]
to mediastinal nodes irrespecti[illegible]
extreme right indicates the pr[illegible] [illegible]ective of
presence or absence of metastases) to cases with metastases alone Metastases means distant metastases while recurrence means tumour tissue arising from the bronchial stump or lymphnodes in the hilus or mediastinum

TABLE 5
Necropsy Findings in Relation to the Histological Type

	Dead of lung cancer	Recurr alone	Recurr + metast	Total recurr	Metast alone	Recurr metast
Squamous cell carc para keratotic group	32	13	15	28	4	7/1
Squamous cell carc poly morphocell group	15	6	7	13	2	6/1
Undifferentiated carc	12	1	9	10	2	5/1
Adenocarcinoma	5	0	3	3	2	2/1
Total number of resected cases dead of lungcancer	64	20	34	54	10	5/1

Correlation between Operative Findings and Autopsy Findings

Such a correlation has only been made for the 119 patients who died later than two months after operation. The relative figures are given in Tables 4 and 5. The figures in the column to the extreme right express the relation between patients with local recurrences (irrespective of wether there were simultaneous metastases or not) and those with distant metastases only. As was expected, the figures show that with increasing local extend and lymphogenous infiltration a similar increase is seen in the frequency of local recurrences, particularly so in respect of the group C tumours, which all, with the exception of one case, showed local recurrence.

The figures in Table 5 show that undifferentiated carcinoma and adenocarcinoma metastazise more frequently than squamous cell carcinoma, but that also in undifferentiated carcinoma the metastases usually occur in connection with local recurrence. This phenomenon is least pronounced in the group of adenocarcinoma.

DISCUSSION

Bell, Gibbons & Tolstedt (1963) performed explorative laparotomy in 30 patients with bronchogenic carcinoma and found distant metastases in six of these patients.

Borrie (1952), in a material of 28 patients who died postoperatively after thoracotomy for bronchogenous carcinoma, found distant metastases which had not been diagnosed at the time of operation in four patients (14 per cent).

In the present material it was found that among patients who died within the first two months after operation 14 per cent had distant metastases. It must be assumed that the vast majority of these metastases were already present at the time of operation, which is in good agreement with the observations made by *Bell et al* (1963), and by *Borrie* (1952).

The general impression received from literature is that lungcancer metastazises frequently and extensively. In the necropsy series here referred to (*Ask-Upmark* 1932, *Ochsner & DeBakey* 1942, *Fried* 1948, *Kahlau* 1954, *Callum & Payne* 1955, *Budinger* 1958, *Willis* 1960, *Onuigbo* 1961), which are all dominated by non-operated patients, the frequency of metastases is between 60 per cent and 75 per cent.

The findings derived from the present series of lung resections show a corresponding frequency of distant metastases (69 per cent), which is in good agreement with *Aylwin* (1951), who by comparing operated with non-operated cases of lungcancer found distant metastases in 60 per cent and 70 per cent, respectively. However, if these metastases appear in connection with local recurrence, the small difference between the two groups must be explained by the fact that concerning the ability to produce metastases the local recurrence must behave like a primary tumour.

The results of autopsy in the present study do in fact show that the metastases usually occur in connection with local recurrence. Distant metastases as the only tumour recurrence were only found in 16 per cent of the patients. From the observations by *Bell et al* (1963) and by *Borrie* (1952) as well as from the post mortem results of the present material of patients who died from post-operative complications within the first two months after operation, we know that clinically unrecognized metastases were present in a similar number of patients at the time of operation. Since metastases in none of these cases were the primary cause of death, the frequency of their presence must have been the same in patients who survived the first two months. This permits of the assumption that in the group of patients where distant metastases were the only tumour manifestation the majority of these metastases must have been present prior to the operation. In other words the results of this investigation indicates that when distant metastases are found in patients following resection for lung cancer, their presence can be considered either secondary to a local recurrence or be explained by the fact that they were already present but undiagnosed at the time of operation. Accordingly this study gives no support to the view that tumour cells circulating in the peripheral blood at time of operation set up unexpected metastases in patients otherwise radically treated. This is in good agreement with the observation made by *Engell* 1959 that the demonstration of tumour cells in the vascular system during operation is of no certain prognostic significance.

The present observations must be taken with the reservation that the figures are small and only two thirds of the patients underwent necropsy examination.

SUMMARY AND CONCLUSION

An account is given of the autopsy results in 133 patients following resection of the lung for bronchogenic carcinoma. 84 per cent of the

TABLE 5
Necropsy Findings in Relation to the Histological Type

	Dead of lung cancer	Recurr *alone*	Recurr + metast	Total *recurr*	Metast *alone*	Recurr *meta t*
Squamous cell carc para keratotic group	32	13	15	28	4	7/1
Squamous cell carc poly-morphocell group	15	6	7	13	2	8/1
Undifferentiated carc	12	1	9	10	2	5/1
Adenocarcinoma	5	0	3	3	2	2/1
Total number of resected *cases dead of lungcancer*	*64*	*20*	*34*	*54*	*10*	*5 1*

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Such a correlation has only been made for the 119 patients who died later than two months after operation The relative figures are given in Tables 4 and 5 The figures in the column to the extreme right express the relation between patients with local recurrences (irrespective of wether there were simultaneous metastases or not) and those with distant metastases only As was expected, the figures show that with increasing local extend and lymphogenous infiltration a similar increase is seen in the frequency of local recurrences, particularly so in *respect of the group* C *tumours, which all, with the exception* of one case, showed local recurrence

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In the present material it was found that among patients who died within the first two months after operation 14 per cent had distant metastases It must be assumed that the vast majority of these metastases were already present at the time of operation, which is in good agreement with the observations made by *Bell et al* (1963), and by *Borrie* (1952)

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METHOD FOR THE IDENTIFICATION OF THE ORIGIN OF FIBRINOID AND OF OTHER INSOLUBLE PROTEINS

By

A Lehtonen, V Näntö and E Kulonen

Received 24 ii 64

Several chemical origins of fibrinoid¹ have been suggested blood proteins with emphasis on fibrinogen or γ globulin, degenerated collagen or precipitated ground substance including the acid mucopolysaccharides (*Clark, Graef & Chasis* 1936, *Bahrman* 1937, *Klemperer, Pollack & Baehr* 1942, *Altschuler & Angevine* 1949) The most recent opinions are based on results obtained with 'fluorescent antibody - technique and favour the oldest ideas *i e*, fibrinogen as the main source of fibrinoid (*Gitlin, Craig & Janeway* 1957), although also γ globulin has been demonstrated in the rheumatic noduli with the same technique (*Vazquez & Dixon* 1958)

The purpose of this paper is to describe a fresh chemical approach which is applicable on all insoluble proteins of obscure origin This procedure is modified from the 'fingerprint method of *Ingram* (1956, 1958) and *Katz, Dreyer & Anfinsen* (1959) Every protein contains an unique peptide pattern which can be visualized and identified Ingram has demonstrated variations between similar haemoglobin proteins, which differ only by a few or even by a single amino acid in the peptide sequence In this work the peptides were obtained by hydrolysis of the materials with trypsin Several other proteolytic enzymes were tried also and they may be suitable for other objects

EXPERIMENTAL

Hydrolysis The tissue samples were homogenized in water with P[illegible] homogenizer and the insoluble [illegible]

[illegible] ples [illegible] few

We a[illegible]
Reuma[illegible]
The rh[illegible]
M D R[illegible] Hospital Heinola, Finland The γ globulin and plasma albumin were gifts from State Serum Institute Helsinki

patients died from local recurrence, frequently in connection with distant metastases 16 per cent died from distant metastases without local recurrence On the basis of the literature and the results of this study it is suggested that in the latter cases the metastases were present prior to the operation in a clinically unrecognized form In other words patients resected for lung cancer does not develop metastases *after the operation* unless there is a local recurrence to explain their origin

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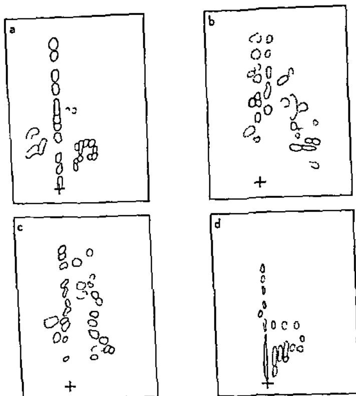


Fig 2 a–d

The peptide patterns of some trypsin digested proteins treated similarly as the rheumatic noduli a) human fibrin clot b) human plasma γ globulin c) human plasma albumin d) rat tail tendon collagen

This method is suitable for the study of also other pathological substances *e g*, of amyloid or hyalin Difficulties arise, when the proteins are mixtures but in principle, even then the origins could be identified by some unique peptide spots The number of expected peptides is the same as the number of arginine plus lysine in a molecule plus one However, in large molecules as in fibrin, the number of peptide spots is no more useful for appraisal of the proper and complete enzymatic action The eventual large peptides yield rather faint colours with ninhydrin

SUMMARY

The "fingerprint"-technique of *Ingram* was applied to the chemical identification of fibrinoid material from the rheumatic noduli It is concluded that the trypsin digested matter resembled fibrin but not γ globulin, plasma albumin or gelatin

drops of toluene were used to prevent the microbial growth The mixture was kept at +37° C for 24 hrs with continuous shaking and the pH was adjusted if necessary to pH 8 2–8 5 by addition of ammonium hydroxide

The nondigested material was precipitated with 10 per cent (w/v) trichloroacetic acid (*Clotten & Clotten* 1962) and the supernatant cleared by centrifugation The trichloroacetic acid was removed by repeated extractions with ether The buffer salts are volatile and the samples could be applied on paper without any further treatment

Preparation of the Fingerprint Map of the Peptides The two dimensional separation was achieved by electrophoresis in one direction and by subsequent chromatography The electrophoresis was done by the method of *Gross* (1956) using Whatman No 3 MM paper, size 30 × 60 cm and pyridine acetate buffer, pH 3 4 (pyridine 17 ml glacial acetic acid 170 ml distilled water *ad* 5000 ml) and a voltage gradient of 40 V/cm The system was cooled to +1° C and the paper was insulated from the cooling blocks by 0 15 mm polythene sheets After a run of 60 min the paper was dried at room temperature The most basic spots travelled in these circumstances ca 20 cm toward the cathode and the most acid ca 5 cm toward the anode from the origin

The chromatography was performed in conventional descending system with *n* butanol acetic acid-water (4 1 5 by volumes) as solvent The fastest spot travelled ca 45 cm

The peptides were stained by dipping the sheets into 0 2 per cent (w/v) ninhydrin solution in acetone The colours were allowed to develop at room temperature The "maps" so obtained were quite reproducible

RESULTS

Peptide patterns of fibrinoid, gelatin and some blood proteins are shown in Fig 1 and 2 The pattern of fibrinoid, extracted by tryptic hydrolysis from rheumatic noduli, and the respective pattern of clotted fibrin agree quite reasonably The differences can be explained by the presence of small amounts of other proteins in the fibrinoid material

It has been demonstrated that trypsin is useful to remove the fibrinoid from the rheumatic noduli (*Ziff, Kantor, Bien & Smith* 1953) although others claim that it is resistant (*Glynn & Loewi* 1952) This difference of opinions may be due to different methods In our hands, the regularly stained fibrinoid material disappeared from microscopical slices after the treatment with trypsin

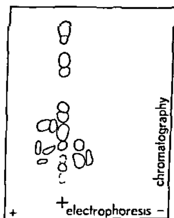


Fig 1

The peptide pattern of trypsin digested material from rheumatic noduli

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From the Medical Department for Non Ruminants and the Department of Anatomy Histology and the Laboratory for Electron microscopy, Royal Veterinary College Stockholm

ULTRASTRUCTURAL ALTERATIONS IN SINUSOIDAL ENDOTHELIUM OF LIVER AND BONE MARROW IN DOGS WITH EXPERIMENTAL HEPATITIS CONTAGIOSA CANIS[1]

By

GERT LINDBLAD & NILS BJÖRKMAN

Received 27 ii 64

The permeability of the endothelial wall has been studied by *Grotte* (1956), who found that dextran molecules with a radius of 35–45 Å could pass through the blood lymph barrier He also postulated ' leaks' with radii of about 135 Å, being especially numerous in the liver In a review *Renkin & Pappenheimer* (1957) dealt with the exchange process and pointed out the significance of hydrodynamik bulk flow for big molecules such as plasma protein *Linblad et al* (1964) have shown a circulatory disturbance in connection with experimental Hepatitis contagiosa canis (H c c) They observed an increased arteriolo venular flow ratio and, in some capillaries complete cessation of flow The histopathological picture of H c c has been extensively described by *Rubarth* (1947) and later by *Innes* (1949) They demonstrated lesions of varying degree in the endothelial nuclei, and intranuclear inclusion bodies Red cells were also found in the space of Disse Ultrastructural alterations in the sinusoidal wall have been reported in liver injuries such as aller-gic liv [illegible]
viral h [illegible]
struct [illegible]
marro [illegible] dogs and dogs with experimentally produced H c c

MATERIAL AND METHODS

[illegible]

[1] Supported by grants from Anslag for framjande av medicinsk forskning vid Veterinarhogskolan

[2] The virus material used was an H c c strain (Titre 7 5 $TCID_{50}$/ml) obtained from R Salenstedt V M D National Bacteriological Laboratory Stockholm 4 ml of an undiluted suspension of virus were inoculated intraperitoneally in each dog

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F g 1

S nuso dal wall from normal l er of a dog Fndothel al sheet (top) sp ace of D se (m ddle) and part of a parenchymal cell (bottom) Ele tronm crograph as in the follow ng p ctures × 24 000



F g 2

RESULTS

After inoculation the dogs fell ill with varying degree of apathy anorrhexia thirst and a temperature rise Two of the dogs (nos 53 and 54) were very little affected and showed no elevation of the transaminas s The results from the G O T and the O C T determinations are brought together in Table 1

Liver *Ultrastructural Observations*

Normal sinusoids The endothel um formed a thin cytoplasmic layer covering the parenchymal microvilli (Fig 1) Occasionally there were

needle under thiopentone sodium anaesthesia One dog (No 24) died 5 days after inoculation The autopsy findings were typical of H c c [1] After 32 59 and 78 days when complete clinical recovery was observed samples were taken from the dog (No 22) that had shown the most severe degree of illness Biopsy specimens were also taken from a healthy dog for controls A further experiment was carried out on 4 dogs with experimental H c c [2] Preinoculation samples were taken for controls and frequent sampling was done until clinical recovery (see Table 1) The course of the disease was followed by frequent examinations of body temperature and the plasma activity of *glutamic oxalacetic transaminase (G O T) and ornithine-carbamyl* trans ferase (O C T) (Table 1) (cf *Lindblad & Persson* 1962) From 2 of the dogs in the latter group (Nos 51 and 52) bone marrow samples from the ribs were taken at the same time, using technique described by *Lindblad & Baelgren* (1964) Pieces of ribs were split into 4 parts to obtain access to the bone marrows which were cut into small pieces with a razor blade The material from the liver and bone marrow was immediately immersed in buffered osmium tetroxide solution (*Millonig* 1962) and fixed for 2 hours The specimens were embedded in Epon (*Luft* 1961) For identification of sinusoids large sections 1 µ in thickness were prepared and stained with buffered toluidine blue solution (*Bjorkman* 1962) Areas free of mechanical damage were selected and the blocks were trimmed Thin sections were prepared stained and examined in a Siemens Elmiskop I at 60 kV

TABLE 1

G O T and O C T Determinations from Dogs with Experimental H c c

Dog no		Days after inoculation 0	2	4	6	8	9	11	12	14	16
21	G O T	23 5	27	40§	38 5§		21 5§		12		19 5
	O C T	0	3 4	6 1	5 5		2 8		1 6		1 1
22*	G O T	31	101	72§	83§		30§		45		27
	O C T	4 4	50 5	20 8	17 3		3 4		20 5		8 0
23	G O T	25 5	57 5	88§	134§		30§		26		18
	O C T	1 7	16 1	20 2	40 2		1 6		2 8		1 7
24	G O T	21 5	72 5	510§	Dead						
	O C T	1 6	23 5	98 5							
25	G O T	27	28	48 5§	53 5§		17§		14		13
	O C T	1 7	6 6	13 9	12 7		4 4		4 5		1 6
51	G O T	22 5§†	15§†	21 5§†	38§†	27§†		19§†		15§†	
	O C T	1 0	3 3	5 6	11 0	1 6		0 6		0	
52	G O T	24 5§†	18§†	49§†	60§†	72 5§†		25 5§†		15§†	
	O C T	2 6	1 6	3 0	3 7	28 5		1 7		1 5	
53	G O T	16§	12§	21 5§	26§	17§		14 5§		16§	
	O C T	2 0	2 5	1 0	1 9	4 3		0 5		3 8	
54	G O T	14§	16§	14§	16 5§	16 5§		12§		13 5§	
	O C T	2 1	0	1 0	2 5	2 3		3 7		3 7	

* See the text

§ = this day biopsy specimens of the liver were taken for electron microscopical examinations

† = this day biopsy specimens of bone marrow were taken for electron microscopical examinations

[1] The autopsy was carried out at the Department of Pathology Royal Veterinary College Stockholm

[2] The virus material used was an H c c strain (Titre 7 5 TCID$_{50}$/ml) obtained from B Salenstedt V M D National Bacteriological Laboratory Stockholm 4 ml of an undiluted suspension of virus were inoculated intraperitoneally in each dog

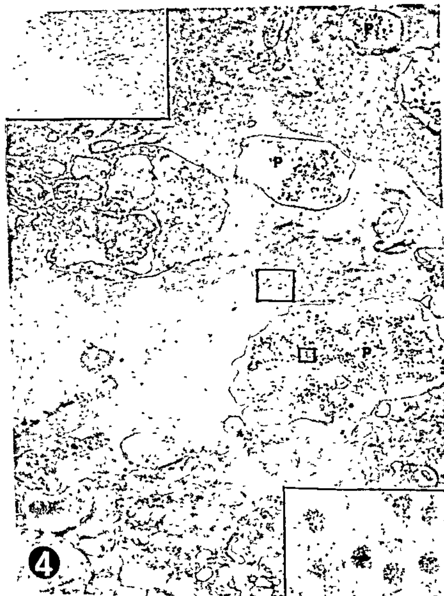


Fig 4

Hepat[illegible]
the
disa
of n
partic[illegible] (mark
fibrin clots (m
right × 18 000



F q 3

Hepat c sinus dal wall v ith fragmentation f the endothel al cytoplasm caus ng oj en commun cation l etv een the s nuso dal lumen and the space of D sse Note the ab indance of blood plasma in the l men (top left and the aggregat ons of ribo- omes nto dense part cles in the parenchymal cell bottom ri_ht 4 la s after inoculation × 24 000

also openings in the endothelial layer seemingly permitting free com munication between the sinusoid and the space of Disse In rare cases defects in the wall occurred which were due to mech inic il damage When Kupffer s cells occurred the sinusoidal wall was much thicker than when it consisted of ordinary endothelium

Sinusoids from inoculated dogs In samples taken 2 days after ino

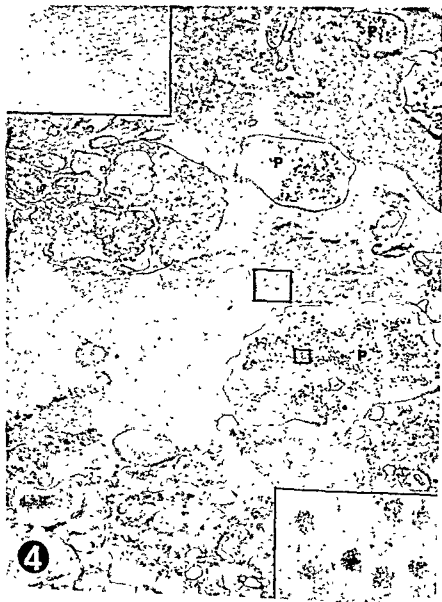


Fig 4

Hepat... the d... disap... of m... parti... fibri... right

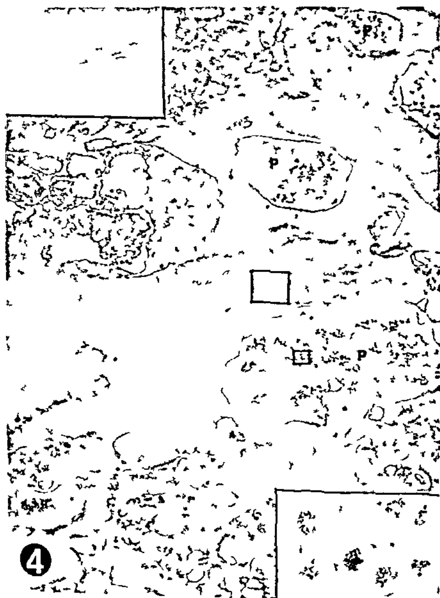


Fig. 3

Hepatic sinusoidal wall with fragmentation of the endothelial cytoplasm causing open communication between the sinusoidal lumen and the space of Disse. Note the abundance of blood plasma in the lumen (top left) and the aggregations of ribosomes into dense particles in the parenchymal cell (bottom right). 4 days after inoculation × 24 000

also openings in the endothelial layer seemingly permitting free communication between the sinusoid and the space of Disse. In rare cases defects in the wall occurred, which were due to mechanical damage. When Kupffer's cells occurred the sinusoidal wall was much thicker than when it consisted of ordinary endothelium.

Sinusoids from inoculated dogs. In samples taken 2 days after ino-



F g 4

H pa nu o d f om the fatal case taken 4 days after noc
h dog]] Th
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ght corner × 200 000



Fig 5

Sinusoid from the liver of a dog 28 days after inoculation when the dog had recovered clinically and the endothelium had been restored The wall contains a Kupffer cell (middle) and the lumen contains red cells (left) Note lipid droplets (l) in a parenchymal cell × 6 000

culation, no differences from the normal material were observed In samples taken from 4 to 11 days after inoculation, intact sinusoids were observed as well, but in many cases, the endothelium was damaged The lesions varied from slight defects in the cytoplasmic sheet to complete disappearance of the endothelium with denudation of the entire surfaces of the parenchymal cells Most alterations observed, however, were moderate although obvious Slight alterations were seen in some endothelial cells which still showed no defects These cells had swollen cytoplasm and swollen mitochondria, although the parenchymal mitochondria were still unchanged (Fig 2) In other cells there were swelling and fragmentation of the endothelial cytoplasm into rounded or irregular lumps offering free communication between the sinusoid and the space of Disse (Fig 3) This severe type of lesion was the most characteristic change in the affected livers In connection with these endothelial alterations, lesions of parenchymal cells were also observed, mainly in the form of the occurrence of lipid droplets The most severe damage consisted in a loss of endothelium and destruction of parenchymal cells The sinusoidal lumen then contained clots of fibrin (Fig 4)

Processes of Kupffer's cells containing dense round particles of a uniform size of 30 mμ were observed

The above-mentioned alterations were on the whole correlated to the degree of severity of the illness as it manifested itself by clinical symp-



F g f
Double lay r s nuso dal end thel um co er ng a m eloc te from bone marro of a normal dog Note caveolae (arro s) and p nocytot c v sicles n the endothelial yto plasm × 30 000

toms and transaminase elevations Thus the fatal case (no 24) pre sented very severe damage (Fig 4) Less severe lesions were found in dog no 29 which was the sickest among the surviving cases In dogs nos 53 and 54 the alterations were insignificant In specimens taken 14 days after inoculation or later there were no or insignificant alter at ons in the endothelium although parenchymal cells were found wh ch had not restored their normal ultrastructure (Fig 5)

Bone Marrow

Normal sinusoids The endothelium formed sheets wh ch were ge nerally thicker than those found in the liver and in many cases two endothel al layers occurred (Fig 6) In most cases the endothelium was intact although defects were also seen presumably artifacts caused at the preparation

Sinusoids from inoculated dogs As in the liver the sinusoids ap peared unaltered 2 days after inoculation Endothelial lesions and a subsequent restoration were observed The course of events ran ap proximately parallel to that observed in the liver As in the liver there was fragmentation of the endothelial cytoplasm (Fig 7) However a complete loss of endothelium was noted in many cases and it was therefore hazardous to determine what would actually represent a si nusoid After 14 days the endothelium was restored and had the same appearance that in pre noculated cases (Fig 8)

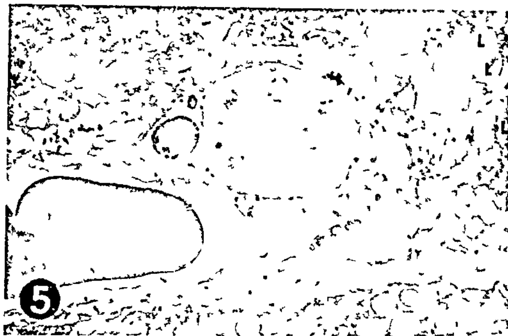


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Alterations were not observed 2 days after inoculation, neither in the liver nor in the bone marrow At this time the dogs from which samples were taken had a rise of temperature but no other signs or affection After 4 days alterations were observed in most cases, and co existing clinical symptoms and increased transaminase values were observed Restoration of the endothelium and clinical recovery were noted after 14 days

However, the endothelial alterations vary in appearance and intact endothelium is present even in affected organs Thus, a strict synchronism between the morphological and the clinical changes cannot unreservedly be inferred It is worthy of note that alterations in parenchymal liver cells were seen after the sinusoids had been restored and the dogs had recovered

The changes in the sinusoids in viral human hepatitis have been described as a narrowing or disappearance of the space of Disse (*Cossel* 1959) or as alterations in Kupffer s cells (*Miyai et al* 1963) In H c c we have found the main alterations to be swelling fragmentation, and ballooning of the endothelial cytoplasm A similar alteration of the microvilli has been reported in allergic injury of the liver (*Steiner* 1961) Although we found a fragmentation of the bone marrow endothelium the sinusoidal wall was often absent This suggests that the injured endothelium has been swept away by the blood stream, and that hence the anchoring of the sinusoidal wall is weaker than in the liver The particles found in processes of Kupffer s cells in the most severily affected dog were uniform in size, shape, and density, which might suggest their viral nature The following dimensions observed under different conditions have been reported 55–65 mμ (*Tajima & Holokoski* 1958 *Tajima et al* 1961), 57 mμ (*Tournier et al* 1960) 82 mμ (*Davies et al* 1961) *Carmichael* (1962) has reported particles of three classes 55–60 mμ 35–40 mμ, and 22–28 mμ As we observed particles in only one case no definite conclusion can be drawn

There is a normal permeability of the endothelium to particles (cf *Grotte* 1956) but this does not necessarily mean that there are openings in the wall (*Bennett et al* 1959) although normal opening in the sinusoidal wall have been suggested for instance by *Steiner* (1961) H

SUMMARY

Biopsy specimens from the liver (10 cases) and bone marrow (2 cases) from dogs with experimentally produced H c c were investigated with the electron microscope to study the influence of H c c on sinusoidal endothelium In affected dogs sinusoids showed the following alterations Swelling of the endothelial cytoplasm fragmentation of the cytoplasm and loss of endothelium In a fatal case, dence round



Fig 7
S nus i l with fragmented endotl elium from l one marrow of a log 4 days after in culation × 1? 500



Fig 8
Doul le layer s nu oi lal en lothel m from bone marrow of a recovered dog 28 da s after inoculat on × 18 000

DISCUSSION

The ultrastructure of the sinusoidal endothelium of normal dog livers presented features that have already been described in other species by e g *Fawcett* (1955) and *Cosset* (1959 a and b) and will not be dis cussed here Corresponding endothelium from bone marrow has pre viously been studied with the electron microscope by *Zamboni & Pease* (1961) Therefore our recording of normal endothelium serves only as reference for evaluation of the pathological cases

The methods of taking biopsy specimens involve risks of mechanical damage and in rare cases defects due to the preparation were observed in the liver material However such artifacts could easily be distin guished from the typical alterations attributable to the illness As re gards the bone marrow artifacts were more frequent but the bone spicu les supported the soft tissue and if the blocks were trimmed between adjacent spicules artifacts were mostly avoided

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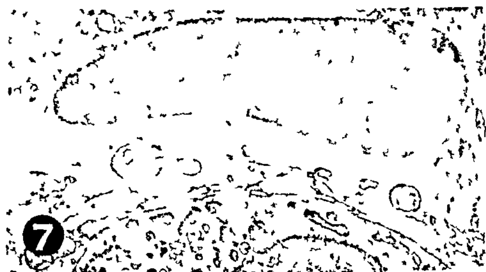


Fig 7
Sinus id with fragmented endothelium from bone marrow of a dog 4 days a in oculation × 12 500



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CHOICE OF STAINING METHOD FOR DETERMINATION OF THE PERCENTAGE OF EPITHELIUM IN THE CHICK THYROID

By

PETER WAHLBERG

Received 03 iii 64

Young cockerels have been regarded by several authors as suitable for thyroid studies, and especially for TSH investigations The histological studies which previously were predominant have largely been replaced by other methods The histological evaluation of thyroid activity is still important, however, as one of the few methods which give a permanent and reproducible picture of some aspects of thyroid function

Histological studies on the height of the acinar epithelial cells of the chick thyroid have been carried out by a number of workers (1, 5, 6, 8) The appearance of large colloid droplets in the acinar cells has been the subject of studies by *Dvoskin* (2) For assay purposes, this author compared the effect of TSH upon this reaction with the effect upon acinar cell height and thyroid weight in chicks He found that by the colloid droplet method a dose of TSH could be demonstrated which was 1/40 of the smallest dose that could be demonstrated by the cell height method

Lotila & Kannas (10) have elaborated a method for estimation of the percentage of epithelium in the thyroid by linear measurement of epithelium colloid and stroma in histological sections The method has been further elaborated for assay purposes in guinea pigs by *Tala* (9) It has proved especially valuable in experimental work

Several fixing and staining methods can be used for the determination of the histological activity of the chick thyroid The choice of method depends upon which histological reaction is to be studied For the colloid droplet method *Dvoskin* (2) fixed his preparations in Carnoy s fluid for one hour, and stained them with his own modification of *de Robertis* (7) version of the Heidenhain azan stain In this modification the preparations are kept in phosphotungstic acid for only one hour With this stain, the colloid becomes intensely blue, and intracellular colloid droplets are clearly visible The epithelium is only slightly stained but the contours of the epithelial cells are clearly visualized

particles with uniform size of 30 mμ were found in processes of Kupffer's cells It is concluded that the damage on the endothelium increases the permeability to large particles

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MATERIAL AND METHODS

The material comprised 32 White Leghorn cockerels To obtain variation in thyroid activity the animals were each given a subcutaneous injection of 0 125 USP units of TSH This dose is above the upper limit for increased activity (4) Groups of chicks were sacrificed before and at 1 2 4 and 8 hours after the injection The thyroid glands were removed and from each animal one thyroid lobe was fixed for 1 hour in Bouin s fluid the other lobe for 1 hour in Carnoy s fluid The lobes were embedded in paraffin and sectioned at 3 μ The lobe fixed in Bouin s fluid was stained by *Koneff's* (3) modification of the Mallory azan stain whereas the lobe fixed in Carnoy s fluid was stained by Dooskin s (2) modification of *de Robertis* (7) version of the Heidenhain azan stain The sections were projected through a microscope on a plain white surface and measured according to the method of *Uotila & Kannas* (10) and *Tala* (9) as described earlier (11) For projection an objective lens ×20 was used the ocular was × 15 The linear amplification was increased by the distance to the surface which was 50 cm From each lobe 4 sections were measured the total length measured being thus 160 cm *per* lobe

The mean value and the standard error of the mean (e) were calculated from each group of chicks with both staining methods The standard error of the difference (e_d) and the factor t and P values obtained by comparing the means of the different groups were also calculated

RESULTS AND DISCUSSION

The results are shown in Table 1 and Fig 1 There is a good correspondence between the mean values of the results obtained by the two methods in each time group This is seen even more clearly in Table 2 The statistical data show that the apparent differences of the mean values given by the two methods in each time group are not significant Thus the results given by the two staining methods can be regarded as belonging to the same material, and they can be placed in the same series of observations This has been done in Table 3, which is based on the total number of observations from both thyroid lobes of each chick The total length measured from each animal is thus 320 cm The table shows a good correlation between the present data and the results of previous time-response investigations (4, 11)

TABLE 2

Statistical Relations Between the Results by the Two Staining Methods in Each Time Group

Time after TSH hours	e_1	t	P	$t_{P=0\,1}$*
0	3 3	0 39	>0 1	1 782
1	5 8	0 019	>0 1	1 860
2	5 2	1 05	>0 1	1 812
4	3 4	0 80	>0 1	1 782
8	4 4	0 50	>0 1	1 782

* $t_{P=0\,1}$ = the value of t if P = 0 1 at the corresponding degree of freedom

Objections may, of course, be raised to the small number of animals examined However, it must be noted that the e values in the different groups are of the same magnitude as those found in earlier

Uotila & Kannas' (10) histoquantitative method for determination of the percentage of epithelium has been used in chick studies by, for instance, *Lamberg* (4) and *Wahlberg* (11) When this method is used it is important to obtain a good contrast between colloid and epithelium For this reason, preparations have been fixed in Bouin's fluid and stained by *Koneff's* (3) modification of the Mallory azan stain With this stain, the colloid becomes blue and the epithelium red Thus, this stain is well suited for the histoquantitative method

When the *Koneff* (3) stain is used, it is not possible to obtain exact information about the intracellular colloid droplets of the chick thyroid Hence, it was decided to test the suitability of the *Dvoskin* (2) stain for the measurement of the percentage of epithelium by *Uotila & Kannas'* (10) method, and a study was carried out in which comparable preparations of both types were used

TABLE 1
Percentage of Epithelium

Time after TSH hours	Number of animals	Bouin + Koneff			Carnoy + Dvoskin		
		Number of lobes	Mean	e	Number of lobes	Mean	e
0	7	7	55 2	2 25	7	56 5	2 60
1	5	5	49 0	3 58	5	50 1	4 70
2	6	6	62 6	3 10	6	57 1	3 65
4	7	7	66 3	2 26	7	63 6	2 49
8	7	7	73 0	3 25	7	75 2	3 00

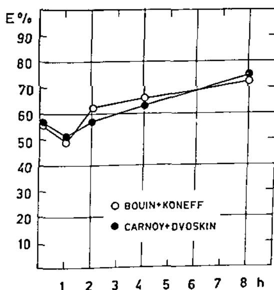


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Percentage of epithelium (E%) Time response relations with both methods

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Time after TSH hours	e_d	t	P	$t_{P=0.1}$*
0	3.3	0.39	>0.1	1.782
1	5.8	0.019	>0.1	1.860
2	5.2	1.05	>0.1	1.812
4	3.4	0.80	>0.1	1.782
8	4.4	0.50	>0.1	1.782

* $t_{P=0.1}$ = the value of t if P = 0.1 at the corresponding degree of freedom

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4	7	7	66 3	2 26	7	63 6	2 49
8	7	7	73 0	3 25	7	75 2	3 00

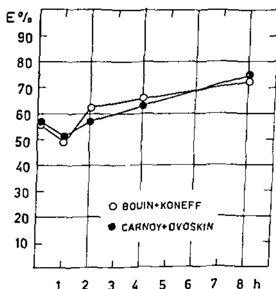


Fig 1

Percentage of epithelium (F%) Time response relations with both methods

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investigations (11) (see Table 1) As already mentioned, pairs of slides were made with the two methods from the same animal, the same degree of stimulation for each pair being thus ensured

TABLE 3

Percentage of Epithelium Time Response Relations Whole Material

Time after TSH hours	Number of lobes	Mean	e	P_o*	P_a*
0	14	55 85	1 59	–	–
1	10	49 55	8 71	>0 1	>0 1
2	12	59 85	2 70	>0 1	=0 01
4	14	64 95	1 61	<0 001	>0 1
8	14	74 10	2 12	<0 001	0 01>P>0 001

* P_o = P comparing the group with untreated controls
P_a = P comparing the group with the one treated for the next shorter time

The results show that in investigations of the activity of the chick thyroid by *Uotila & Kannas'* (10) method, under the present test conditions, both the staining methods here employed can be used Thus, one is free to choose between fixation with Bouin's fluid and staining by *Koneff's* (3) modification of the Mallory azan stain, and fixation with Carnoy's fluid followed by staining by *Dvoskin's* (2) modification of *de Robertis'* (7) version of the Heidenhain azan stain Using the latter stain, one can thus choose freely between the histoquantitative method and the colloid droplet method, or use both methods on the same preparations

SUMMARY

32 White Leghorn cockerels were injected with 0 125 USP units of TSH and groups were sacrificed at 0, 1, 2, 4 and 8 hours One thyroid lobe of each animal was fixed in Bouin's fluid and stained by *Koneff's* (3) modification of the Mallory azan stain and the other lobe was fixed in Carnoy's fluid and stained with *Dvoskin's* (2) modification of *de Robertis* (7) version of the Heidenhain azan stain The percentage of epithelium was determined from both preparations by the method of *Uotila & Kannas* (10) The results show that the value obtained does not depend of which of the two fixing and staining methods is used This is convenient, as earlier investigations have shown that *Dvoskin's* (2) stain is to be preferred for studies of intracellular colloid droplets

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tion of the pituitary adrenal system when the active mobility of eosinophilic leucocytes is restricted (*Osada* 1961) The reticuloendothelial system eliminates eosinophilic leucocytes during glucocorticoid effect (*Esselier, Jeanneret & Morandi* 1954) Stress which involves in addition to hormonal factors essentially neural factors leads to the accumulation of eosinopenic metabolites in the organism (*Tanos, Szilasy, Varro, Eisner & Olah* 1953)

The number of eosinophilic leucocytes in the lamina propria of the gastrointestinal canal obviously diminished more rapidly in the gastric mucosa under the influence of adrenocorticotrophin and glucocorticoids (*Rasanen* 1960) than in the mucosa of the small intestine *Sundell* 1958) Tissue eosinopenia is probably caused by catabolic metabolites and its magnitude is dependent on the biologic activity of glucocorticoid (*Rasanen* 1962) The inhibition of the eosinopoiesis of bone marrow during glucocorticoid effect (*Aschkenasy, Abitbol Piette & Layani* 1956) may be a potential factor in the aetiology of tissue eosinopenia

Eosinophilic leucocytes possibly inhibit the biologic effects of histamine (*Kovacs* 1950, *Vercauteren* 1953, 1955, *Archer* 1960) The superficial part of the gastric mucosa contains abundant histamine (*Feldberg & Harris* 1923) and mast cells (*Rasanen* 1958 1960) When the mast cells degranulate, the gastric mucosa loses a great deal of histamine (*Foley & Glick 1962)* The histamine "in statu nascendi which is probably liberated from mucosal mast cells during a short term stress may consume eosinophilic leucocytes on a larger scale in the superficial than in the basal part of the mucosa and then superficial tissue eosinopenia in the gastric mucosa is perhaps greater than the tissue eosinopenia in its basal part

METHOD

Four month old male rats of Sprague Dawley strain were used for the study They were allowed to adapt themselves to laboratory conditions for 10 days in an animal house at a *temperature of 20–22° C* on water and a mixed diet ad libitum They were divided into groups of 10 animals each for the duration of the actual experiment and subjected to the following intoxication and stress conditions

1 Insulin 2 i u per rat (mean weight 177 g)
2 Insulin 2 i u + 0 25 mg of parathion per rat (mean weight 167 g)
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[illegible] g a 400 fold magnification (Leitz microscope plane objective and plane and wide angle eyepiece) the eosinophilic leucocytes were counted in the superficial

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The Department of Pathology, Section II, University of Helsinki, Helsinki

REGIONAL DISTRIBUTION OF TISSUE EOSINOPHILS IN THE MUCOSAL RAT STOMACH UNDER VARIOUS EXPERIMENTAL CONDITIONS, ESPECIALLY DURING SYSTEMIC STRESS[1]

By

TOIVI RASÄNEN

Received 14 iii 64

Tissue eosinophilia in human gastric mucosa is at its most profuse in the basal part (*Hamperl* 1932); the number of eosinophilic leucocytes in gastric mucosa is c 10,000 per cum mm of tissue (*Rasanen* 1958) It has been calculated that c 10 per cent of the eosinophilic leucocytes of the rat organism as a whole are located in the wall of the gastrointestinal canal and that it contains c 30 times the number of eosinophilic leucocytes in the blood (*Rytomaa* 1960)

The number of extravascular eosinophilic leucocytes is increased by foreign proteins and hypersensibilization (*Kultschitzky* 1897, *Biggard* 1932, *Speirs* 1955) Eosinophilic granulocytes proliferate in the gastric mucosa of adrenalectomised rats (*Rasanen* 1961) but not in the mucosa of the small intestine (*Sundell* 1958)

The eosinophilic leucocytes of the tissues are probably of myeloid origin travelling with blood and migrating via the wall of the capillaries (*Weinberg & Seguin* 1914, *Maximow* 1927) The intravascular phase —long distance transport—probably lasts 15-90 min (*White* 1954, *Rytomaa* 1960)

The life of eosinophilic leucocytes is c 10 days (*Osgood* 1937) which presupposes a 24-hour consumption of c 10^8 eosinophilic leucocytes in the rat organism Their accumulation in the blood after resection of the ileum is indicative of their profuse elimination via the mucosa of the ileum (*Teir, Rytomaa & Cederberg* 1963) Morphologic and histo- and biochemical methods have shown that eosinophilic granulocytes are eliminated by diapedesis (*Teir, Rytomaa, Cederberg & Kiviniemi* 1963) Numerous eosinophilic leucocytes that have penetrated the intestinal epithelium have been encountered in the mucosa of the large intestine (*Maximow & Bloom* 1944) Eosinophilic leucocytes are eliminated through phagocytosis in the peritoneal cavity, especially during stimula-

1 Aided with a grant from the *Sigrid Juselius Foundation and the Damon Runyon Memorial Fund* DRG 664 A

tion of the pituitary adrenal system when the active mobility of eosinophilic leucocytes is restricted (*Osada* 1961) The reticuloendothelial system eliminates eosinophilic leucocytes during glucocorticoid effect (*Esselier, Jeanneret & Morandi* 1954) Stress which involves in addition to hormonal factors essentially neural factors leads to the accumulation of eosinopenic metabolites in the organism (*Tanos, Szilasy, Varro, Eisner & Olah* 1953)

The number of eosinophilic leucocytes in the lamina propria of the gastrointestinal canal obviously diminished more rapidly in the gastric mucosa under the influence of adrenocorticotrophin and glucocorticoids (*Rasanen* 1960) than in the mucosa of the small intestine *Sundell* 1958) Tissue eosinopenia is probably caused by catabolic metabolites and its magnitude is dependent on the biologic activity of glucocorticoid (*Rasanen* 1962) The inhibition of the eosinopoiesis of bone marrow during glucocorticoid effect (*Aschkenasy, Abitbol Piette & Layani* 1956) may be a potential factor in the aetiology of tissue eosinopenia

Eosinophilic leucocytes possibly inhibit the biologic effects of histamine (*Kovacs* 1950, *Vercauteren* 1953 1955 *Archer* 1960) The superficial part of the gastric mucosa contains abundant histamine (*Feldberg & Harris* 1923) and mast cells (*Rasanen* 1958 1960) When the mast cells degranulate the gastric mucosa loses a great deal of histamine (*Foley & Glick 1962)* The histamine in statu nascendi which is probably liberated from mucosal mast cells during a short term stress may

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METHOD

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2 Insulin 2 i u + 0 25 mg of parathion per rat (mean weight 167 g)
3 Parath
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The insulin (Insulin Medica) and ACTH (Cortrophin Organon) were injected in aqueous solution intramuscularly and the ethanol solution of commercial parathion intra abdominally

The rats were killed by decapitation at the times shown in Table 1 The abdominal cavity was opened immediately the stomach was removed and opened its contents emptied carefully and spread on a paperboard strip Fixation was performed in Bouin s solution Paraffin sections 4 μ in thickness were cut as perpendicularly as possible to the mucosa and stained with haemalum eosin after removal of the paraffin

Using a 400 fold magnification (Leitz microscope plane objective and plane and wide angle eyepiece) the eosinophilic leucocytes were counted in the superficial

and basal layer of the corpus mucosa over a length of 3 5 mm from sites correspond ing to one another The means and their standard error are given in the results The significance of the differences of the means was obtained from Fisher and Yates's "Statistical Tables for Biological Agricultural and Medical Research" (1938)

RESULTS

The eosinophilic leucocytes counts per sq mm are shown in Table 1 *The number of tissue eosinophilic leucocytes in the superficial* part of body mucosa decreased after a short period of insulin- or parathion-induced shock The same phenomenon was noted during swimming stress Superficial eosinophilia appeared to decrease less under the influence of adrenocorticotrophin and the difference was not statistically significant No statistically demonstrable change was established in the eosinophil count of the basal part

TABLE 1

The Distribution of Tissue Eosinophils in the Superficial and Basal Part of Rat Gastric Mucosa during the Short-Time Effect of Shock ACTH and Stress

Treatment	Time in minutes	No of cases	Superficial eosinophilia	P	Basal eosinophilia	P <
Insulin 2 i u	*300*	10	*40 ± 10 7*	0 01	*673 ± 86*	
Insulin 2 i u + parathion 0 25 mg	15–30	10	26 ± 13	0 01	624 ± 90	
Parathion 0 125 mg	15 60	10	79 ± 28	0 05	637 ± 87	
ACTH 15 i u	300	10	112 ± 22		665 ± 63	
Swimming stress	*140–230*	*10*	*62 ± 9*	*0 01*	*620 ± 69*	
Controls		10	169 ± 27		891 ± 82	

Fairly numerous leucocytes with annular nuclei and eosinophils that stained poorly in the peripheral protoplasm in the superficial lamina propria of the mucosa were established in the rats given parathion They were obviously eosinophilic leucocytes which had become de-granulated These changes were not demonstrable in the eosinophilic leucocytes in the *basal part of the mucosa*, whose granulation was profuse Eosinophilic leucocyte granulation in the superficial part of the gastric mucosa of the control group rats was normal

The gastric mucosa of the rats given adrenocorticotrophin or only insulin and subjected to the swimming stress displayed a decrease in eosinophilic leucyte granulation on moving towards the surface of the mucosa, *although degranulated* leucocytes with annular *nuclei* were scantier in these cases than in connection with parathion intoxication when the shock was rapid and severe

Eosinophilic leucocytes were not encountered in the lumina of the glandular tubules in the gastric mucosa, nor were they found to migrate via the superficial epithelium into the gastric lumen They were located solely in the lamina propria and muscularis mucosae and here and there in the submucosa

DISCUSSION

Eosinophilic leucocytes appear to undergo a rapid degranulation process in the superficial part of the gastric mucosa during acute shock and stress without any detectable signs of cell destruction in the nucleus of the cell. Eosinophilic leucocytes are obviously destroyed in the basal part of gastric mucosa during glucocorticoid effect, parallel with degranulation there is nuclear fragmentation (*Rasanen* 1962) such as occurs in the blood (Padawer & Gordon 1952).

The superficial part of the lamina propria of gastric mucosa contains a profusion of mast cells which degranulate rapidly during stress. Their number in an earlier study was as follows (*Rasanen* 1963):

	Insulin	Insulin + parathion	Parathion	Swimming stress	ACTH	Controls
Medians	291	390	874	594	511	1042
P <	0.01	0.001		0.05	0.05	

Acetylcholine liberated by an insulin induced vagal stimulation, the disintegration of which is inhibited by parathion, possibly functions as a histamine liberator in gastric mucosa. During gastric stimulation histamine appears in gastric juice and increases in the portal blood (*Silen & Eiseman* 1961). Topical histamine liberation in the superficial part of the lamina propria of gastric mucosa may accelerate the degranulation of eosinophilic leucocytes which probably occurs gradually, while they first undergo vacuolization.

After vagotomy and atropine which inhibit the vagal secretory effect on gastric mucosa the quantity of eosinophil granulocytes increases in rat gastric mucosa (*Teir, Wegelius Sundell, Paavarinne & Kuusi* 1955).

The fairly slight loss of eosinophilic granulocytes of tissue in the superficial part of the mucosa after a large single dose of adrenocorticotrophin may be due to the hormonal reaction in the gastric mucosa producing changes that are slower than those caused by neural stimulation (*Rasanen* 1963). Similar eosinopenia has been induced in human gastric mucosa within 8 hours of a single injection of ACTH (*Siurala, Rasanen & Sundberg* 1959). It occurred in a phase in which the mast cells were probably already in a regranulation phase.

During stress histidine decarboxylase activity *increases in tissues* (*Schayer* 1960) but the histamine detoxicating histaminase also increases especially in intestinal mucosa when the *pituitary adrenal system is stimulated* (*Kahlson* 1956). *The* histaminase histamine system undergoes coupled oxidation, presumably by the action of hydrogen peroxide and catalase which serves as a peroxidase. *Peroxidase* is found in eosinophilic leucocytes (*Vercauteren* 1955, *Rytomaa* 1960, *Archer & Broome* 1963) and the peroxidase content of the tissues depends on their eosinophilic leucocyte content (*Rytomaa & Teir* 1961). The great number of eosinophils in intestinal mucosa and their peroxidase content may cause profuse acetylation of histamine *in it*

and basal layer of the corpus mucosa over a length of 3.5 mm from sites corresponding to one another. The means and their standard error are given in the results. The significance of the differences of the means was obtained from Fisher and Yates's "Statistical Tables for Biological, Agricultural and Medical Research" (1938).

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(*Anrep, Ayadi, Barsoum, Smith & Talaat* 1944). The rat organism is hardly capable of histamine acetylation after enterectomy (*Millican* 1953).

Eosinophilic leucocytes appear to have affinity for phagocytosis of mast cell granules (*Welsh & Geer* 1959) and when they proliferate in tissue containing mast cells the histamine content decreases (*Riley* 1956) In tissue extract histamine binds with arginine (*Archer* 1958) which has been isolated also from eosinophilic leucocytes and possibly has an antihistaminic effect (*Vercauteren* 1953, 1955) Eosinophilic leucocytes probably bind histamine (*Kovacs* 1950, *Vaughn* 1953) and their suspension has an antihistaminic effect (*Vercauteren* 1955, *Archer* 1960 a, b, *Broome & Archer* 1962, *Esch & Taubert* 1963)

Degranulation of gastric mucosal mast cells is obviously accompanied by histamine liberation (*Foley & Glick* 1962) and stimulation of parenchymal cells Eosinophilic leucocytes possibly detoxicate the excess histamine in the lamina propria of the stomach which is released during secretion This process may be significant as a consumer of eosinophilic leucocytes Migration of eosinophilic leucocytes via the epithelium of gastric mucosa like that observed in the mucosa of the ileum (*Teir et al* 1963) was not established in the present work

As regards the function of the gastric parenchyma, the profusion of eosinophilic leucocytes in the gastric mucosa of patients with gastric ulcer and gastric cancer (*Rasanen* 1958) may have an inhibitory effect on gastric secretion by binding and destroying endogenous histamine

SUMMARY

It has actually been observed recently that the elimination of eosinophilic granulocytes appears to take place largely via the gastrointestinal canal Their regional distribution in the gastric mucosa is of interest in this respect Therefore, the distribution was studied by counting the number of eosinophilic leucocytes separately in the basal and superficial part of the gastric body mucosa of the rat after insulin shock, parathion intoxication, adrenocorticotrophin injection and swimming stress

The eosinophilic leucocyte count decreased distinctly in the superficial part of the lamina propria during insulin shock, parathion intoxication and stress The reduction in number was due to their degranulation Superficial tissue eosinopenia was scanty after the administration of adrenocorticotrophin

No demonstrable change in the number of eosinophilic leucocytes occurred during this time in the basal part of the mucosa nor were forms suggestive of their degranulation encountered there

The potential rôle of eosinophilic leucocytes in the elimination of topical histaminemia while eosinophilic granulocytes participate in both the binding of histamine and its enzymatic degradation is discussed

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STUDIES ON MONONUCLEAR CELLS OBTAINED FROM SYNOVIAL FLUID OF PATIENTS WITH DIFFERENT TYPES OF ARTHRITIS. CYTOTOXIC EFFECT ON TISSUE-CULTURED HUMAN FIBROBLASTS

By

HELGE HEDBERG and BENGT KÄLLÉN

Received 10 iii 64

For several years, many authors have speculated whether immunological mechanisms are involved in the pathogenesis of rheumatoid arthritis Rheumatoid arthritis is also generally included in synopses of so called autoimmune diseases (see, for example, the reviews of *Dameshek et al* 1961, *Milgrom & Witebsky* 1962, *Waksman* 1962, *Mackay & Burnet* 1963) Experimental counterpart of rheumatoid arthritis is dubious Thus one of the fundamental postulates by *Witebsky* (1957) and *Milgrom & Witebsky* (1962), *i e* that of a passive transfer of the experimental disease, is not fulfilled

Many studies have demonstrated that the various experimental autoimmune conditions are mediated by cell-bound immunologic mechanisms of the so called delayed hypersensitivity or tuberculin type Transfer experiments in experimental allergic encephalomyelitis (*Paterson* 1960, *Stone* 1961, *Åstrom & Waksman* 1962) and in experimental allergic thyroiditis (*Felix-Davies & Waksman* 1961) have shown that these diseases can be transferred with suspensions of lymph node cells, whereas passive transfer with serum has failed *Waksman & Wennersten* (1963) have reported a successful transfer of experimental adjuvans arthritis in rats by means of lymph node cells, and *Hess et al* (1962) transferred autoimmune nephrosis in rats with lymph node cells

Usually it is not possible to carry out transfer experiments for the purpose of directly studying whether lymphoid cells play any rôle in the pathogenesis of human diseases During the last few years, some different *in vitro* systems have been developed which would make such studies feasible In one of these systems, lymphocytes from the patient are combined with living "target" cells kept in tissue culture In animal experimental autoallergic diseases, such models have worked satis-

The cost of this investigation was defrayed by a grant from the *Swedish Medical Research Council*

factorily Thus *Koprowski & Fernandes* (1962) showed that lymph node cells from animals with experimental allergic encephalomyelitis accumulate around glia cells, kept *in vitro* (contactual agglutination), and cause glia cell lysis *Berg & Kallen* (1963) described the same phenomenon using white blood cells, isolated from peripheral blood Studying experimental allergic thyroiditis in rabbits (*Rose et al* 1963) and rats (*Bjorklund* 1964), a similar cytotoxic effect of lymphoid cells on cultured thyroid cells has been demonstrated A parallel can also be drawn to the experimental homograft situation where host lymph cells *in vitro* can be shown to attack and destroy donor cells (*Govaerts* 1960, *Rosenau & Moon* 1961, and others)

This method was adopted by *Berg & Kallen* (1964) in a study on multiple sclerosis They could demonstrate that mononuclear cells isolated from the peripheral blood of many such patients selectively attack cultured glia cells and exert a cytotoxic effect *Perlmann & Broberger* (1963) described a cytolytic activity of lymphoid cells from patients with ulcerative colitis when added to human foetal colon cells *in vitro*

In the present paper, a similar study has been undertaken using lymphoid cells taken from the synovial fluid of patients with various forms of arthritis *Braunsteiner et al* (1963) described a slight, cytotoxic effect of lymph node cells from five rheumatoid arthritis patients on trypsinized cultured human amnion cells Contactual agglutination was also noted Control lymph node cells lacked this activity However, these authors did not study other types of arthritis

CLINICAL MATERIAL

The clinical material comprised 38 patients listed in Table 1 all patients except four were or had been admitted to the Department of Rheumatology University Hospital Lund The patients were adult if not otherwise stated RA patients with subcutaneous nodules and non rheumatoid cases (see Table 1) were specifically chosen which explains the high proportion of such cases The others were randomly *selected among patients with effusion of the knee joint(s)* *Only effusions* amounting to at least 20 ml were examined The majority of the patients studied—rheumatoid as well as non rheumatoid—had shown effusion of the knee joint for several years repeatedly treated with aspirations and with injections of hydrocortisone No patient had received such injections during the three weeks preceding examination

Rheumatoid Arthritis (RA) and Systemic lupus erythematosus (SLE)

In the classification of RA the criteria proposed by *Ropes et al* (1958) were mainly followed All those labelled classical RA showed a positive sensitized sheep cell (SSC) test and the majority had been consistently positive in this test Included as a classical RA was one juvenile RA Four patients with a negative SSC test were labelled definite RA Three of these patients had been consistently negative in the SSC test the fourth who had been afflicted with the disease for six months had been tested only once

One patient with a typical *Felty s syndrome* was also included

Three patients with systemic lupus erythematosus called *SLE* below—or suspected SLE were studied One showed positive LE cell tests a low complement level of serum and slight leucopenia The second had a doubtful rash intermittent fever carditis, severe anaemia nephropathy and a false positive test for syphilis The

third had intermittent fever nephropathy and a false positive test for syphilis In the two last mentioned cases repeated LE cell tests had been negative All three showed arthritis of the rheumatoid type

The cases of SLE were considered clinically active the RA cases showed a varying degree of activity

Non Rheumatoid Arthritis (See Table 1)

Osteoarthritis three patients

Chronic uro polyarthritis (definition *Olhagen* 1960) three patients Two had sacro iliitis and one had active prostato vesiculitis

Ankylosing spondylitis five patients two of these presenting the juvenile forms All had erosions and/or sclerosis of the sacro iliac joints X ray changes indicating spondylitis were present in four of these patients One juvenile case presented no x ray changes of the spine Clinically all showed signs of involvement of the spine

Atypical polyarthritis two patients with main involvement of the big joints Both had consistently negative SSC tests

joints in hands and feet the terminal joints not being affected

The SSC test as well as the Latex agglutination test were negative in all non rheumatoid patients The latter test—when performed— was negative also in synovial fluid from these patients

The majority of the non rheumatoid cases was considered clinically active

METHODS

Synovial fluid was drawn from the patient and treated with hyaluronidase (120 viscosity reducing units/ml 37° C 15 mins) and heparinized A separation of the white blood cells into mononuclears and granulocytes was made according to the method described by *Walker & Palmer* (1962) for whole blood The synovial fluid

1 3 m ... suspension was made in Parker 199 to give a final cell count of

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mononuclears separated as described above were added (volume 1 ml) to the fibroblast cultures which previously were rinsed with pre warmed Parker 199 Mononuclears were left in contact with the cultures for 15 90 ... incubation at 37° C then removed ...

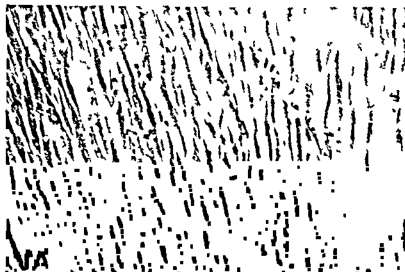
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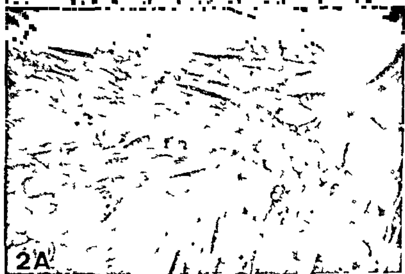
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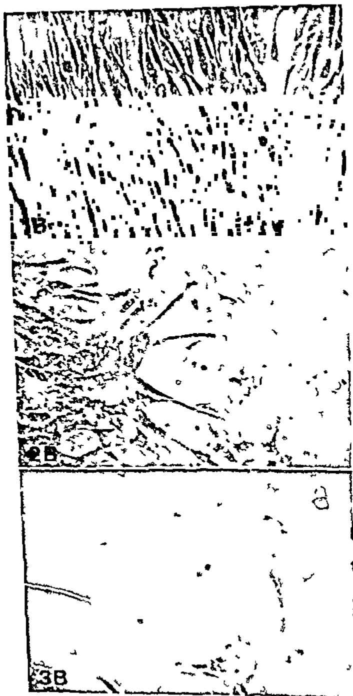
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1A

2A

3A

In Table 1, some data are given of the cases studied together with the outcome of the cytotoxicity tests Repeated tests with mononuclears taken at varying intervals (up to 4 months) from six different patients gave the following results in two patients tests were positive on both occasions, in three negative on both occasions, in one patients (diagnosis psoriatic arthropathy) one test was positive, the second test, 4 months later, being negative

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TABLE 2

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Diagnosis	No of patients	Cytotoxic effect of mononuclear cells only	Cytotoxic effect of mononuclear cells with	
			Patient serum	Blood donor serum
A				
Non rheumatoid arthritis	3	0	0	
Definite RA	2	0	0	0
Class RA	4	0	0	0§
B				
Felty's syndrome	1	0	++	
Definite RA	1	0	++	0
Class RA	1	0	+	0
C				
SLE	1†	++	0	0
Class RA	3	+	0	0
	1	++	0	

* Within each group of patients tests were performed with 25 and with 50 per cent of serum

§ Mononuclear cells from two patients not tested in combination with blood donor serum

† SSC test was positive (see also Table 1)

In Group A, 9 cases are shown in which mononuclears without serum exerted no cytotoxic effect Addition of the patient's own serum or blood donor serum did not influence the outcome of the tests

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but did not seem to influence the outcome of the test, neither did variations in cell counts and percentage of granulocytes

The tests for rheumatoid factor and the estimation of synovial complement (C')activity were performed as previously described by *Hedberg* (1963). Values of the synovial C activity between + 0 20 and —0 20 were considered normal, values below —0 20 were *considered suppressed*

RESULTS

The cytotoxicity tests were scored "0", "+", and "+ +", as shown and described in Figures 1–3 Tests scored "+" and "+ +" were considered positive

TABLE 1

The Cytotoxic Effect of Mononuclear Cells on Human Fibroblasts (without Addition of Serum) in Different Types of Arthritis

SSC = sensitized sheep cell

Diagnosis	No of patients	Positive SSC test	Cytotoxic effect*) 0	+	++
SLE	1	0			1
SLE	2	2			2
Total	3	2		3	
Felty's syndrome	1	1	1		
Classical RA					
A With subcutan nodules	4	4	1		3
B Without subcut nodules	11	11	7	3	1
Total	15	15	8	7	
Definite RA	4	0	4		
Non rheumatoid arthritis §					
A Psoriatic arthropathy	2	0			2
B Other †	13	0	13		

* See Figs 1 3 and text

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† Osteoarthritis 3 patients, chronic uro polyarthritis 3 patients, ankylosing spondylitis 5 patients, atypical polyarthritis 2 patients

Figs 1 2 and 3

Fig 1 "0 A Fibroblast culture before addition ... pic field 20 hours later Phase contrast

Fig 2 +" A Fibroblast culture before addi ... later Phase con ... noted After 20 hours, beginning degener

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In Group C finally 5 cases are shown in which the mononuclears produced a cytotoxic effect which was extinguished by addition either of the patient's own fresh serum or of blood donor serum—fresh or heat inactivated.

In one case of SLE and one of class RA the cytotoxic effect of mononuclears was *not at all* or only partially neutralized by the patient's serum inactivated at 56° C for 30 mins. In one experiment (classical RA) in Group B Table 2 the mononuclear cells were combined with the patient's serum, inactivated at 56° C for 30 minutes. Also here cytotoxicity was provoked.

In some of the cases shown in Table 1 the synovial C activity was also determined. The results are shown in Table 3. In the non rheumatoid controls Group B from Table 1 all three types of tests were negative while in the other groups there is no evident agreement between the three tests.

TABLE 3

The Cytotoxic Effect of Mononuclear Cells (without Addition of Serum), the Synovial Complement (C) Activity and the Sensitized Sheep Cell (SSC) Test in Different Types of Arthritis

Diagnosis	No of patients	Positive SSC test	Synovial C activity: normal	Synovial C activity: suppr	Cytotoxic effect: 0	Cytotoxic effect: +	Cytotoxic effect: ++
SLE	2	2		2			2
	1	0		1			1
Felty's syndrome	1	1		1	1		
Classical RA	5	5	1	4	5		
	3	3		3			3
Definite RA	2	0	2		2		
Non rheumatoid arthritis							
A Psoriatic arthropathy	2	0	1	1			2
B Other	10	0	10		10		

See Table 1

This limited series does not allow for any detailed clinical considerations. Yet it may be *mentioned* that two patients with RA with a relatively fresh effusion (for the past two months) showed a negative cytotoxic test. In one of these in whom the disease had lasted for about six months this test was also performed with the addition of (fresh) serum. In the presence of patient serum but not of blood donor serum the test for cytotoxicity became positive. These two were the only ones in whom the disease had lasted for less than one year. In a third case —of suspected SLE—with an effusion existing for the past half year the test for cytotoxicity (performed only with mononuclears without addition *of serum*) was positive.—There was no connection between on the one hand the result of the cytotoxic test and on the other cell

counts and percentage of granulocytes, the amount of synovial fluid aspirated, or the type of treatment

DISCUSSION

An *in vitro* test has been used for the study of the cytotoxic effect of mononuclear cells obtained from the synovial fluid Although the mechanism underlying the cytotoxic effect is still unknown, parallels drawn with similar phenomena in experimental autoimmune diseases and some clinical entities suggest that the present test system may be of interest As reviewed in the introduction, cytotoxic *in vitro* effects have been described in, for instance, homograft rejection, experimental allergic encephalomyelitis, experimental allergic thyroiditis, multiple sclerosis, and ulcerative colitis

In the present paper, a positive cytotoxic effect has been obtained in tests performed with mononuclear cells from patients with SLE, psoriatic arthropathy, and some cases of RA, whereas tests were negative in all other patients (cf Table 1)

The presence of various immunological disturbances in RA and SLE makes it reasonable to interprete a positive cytotoxic effect as an expression of autoimmunity in these diseases The positive cytotoxic test in the two patients with psoriatic arthropathy is interesting but will not be further discussed

In experimental autoimmune diseases, the pathogenetic importance of sensitized lymphocytes has been demonstrated by transfer experiments (cf introduction) and, the appearance of cytotoxic lymphoid cells in the peripheral circulation before the development of the disease (*Berg & Kallen* 1963) indicates that this phenomenon is due to the immunization procedure By studies of clinical entities, it is impossible at present to state whether the cytotoxicity is primarily involved or it is secondary to tissue damages

In experiments with combinations of mononuclear cells and serum in the tests two different types of effect were seen In five cases, the cytotoxicity of the mononuclears was abolished by addition of serum the patient s own serum or blood donor serum A similar protective effect was seen by *Rosenau & Moon* (1961) when guinea pig serum was added to cytotoxic lymphocytes in tissue culture—In another three cases however the opposite effect was seen Here the mononuclears without serum proved to be non toxic After addition of the patient s own serum strong cytotoxicity developed—blood donor serum had no effect in two of these cases *Govaerts* (1960) obtained an acceleration of a cytotoxic effect by addition of complement, and *Perlmann & Broberger* (1963) needed complement to demonstrate a cytotoxic effect of lymphoid cells

Pulvertaft et al (1959) demonstrated a cytotoxic serum factor in Hashimoto s disease, *Bornstein & Appel* (1961) a myelinolytic serum

factor in experimental allergic encephalomyelitis, and *Berg & Kallen* (1962) a gliotoxic serum factor in the same disease Using our system we have found no indications of a cytotoxic effect of RA serum on human fibroblasts *Fraser & Catt* (1961) gave a preliminary communication on cell damaging effects of RA sera on cultured human synovial cells

Our findings of a protective or potentiating effect of added serum on the cytotoxicity of the mononuclear cells suggest that several serum factors may be of importance in this connection, and that variations in these factors possibly may influence the cytotoxicity of the mononuclear cells *Paterson* (1963) has demonstrated the presence of both cytotoxic (probably 7S) and protective (19S) serum antibodies in rats with experimental allergic encephalomyelitis

More sensitive methods for evaluating cytotoxicity, *e g*, determination of isotope release as suggested by *Perlmann & Broberger* (1963) or studies on growth carried out by *Fraser & Catt* (1961) might perhaps have produced a higher percentage of positive results in the study of mononuclear cells from patients with arthritis

SUMMARY

1 The cytotoxic effect of mononuclear cells from the synovial fluid of patients with various forms of arthritis was *in vitro* tested on human embryonic fibroblasts Cytotoxicity was judged by "contactual agglutination" and fibroblast degeneration Only very marked effects were judged as positive

2 Positive cytotoxicity tests were found in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and two with psoriatic arthropathy Control subjects with non-rheumatoid arthritis of other types were negative

3 No absolute agreement between the outcome of the cytotoxicity test, the sensitized sheep cell test, and the suppression of synovial C'complement, could be observed

4 Parallels are drawn with the presence of similar cytotoxic phenomena in experimental autoimmune diseases and some clinical entities in which an autoimmune pathogenesis has been suggested The possibility that the cytotoxic phenomenon is secondary to tissue damage in the disease, however, is also stressed

5 Addition of serum to the medium at the lymphoid cell test may have different effects A positive cytotoxic reaction can be neutralized by the patient s own, fresh serum or blood donor serum, fresh or inactivated at 56° C for 30 mins In three instances, a negative cytotoxic effect turned positive after addition of the patient s own fresh serum —blood donor serum had no effect in two cases where it was tested In one of these, inactivated patient serum also provoked cytotoxicity

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Acta path et microbiol scandinav 62 189 223 1964

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Head Leiv Kreyberg M D and Patologisk Anatomisk Laboratorium
Ullevål Sykehus Oslo Head Kristen Arnesen M D

EXPERIMENTAL PLATELET AND COAGULATION THROMBI

A Histological Study of Arterial and Venous Thrombi of Varying Age in Untreated and Heparinized Rabbits

By

LEIF JORGENSEN

Received 16 v 64

The concept of thrombosis as an intravital and intravascular formation of a body derived from blood constituents was introduced by *Virchow* (1856) Later *Zahn* (1891), *Aschoff* (1892), and *Welch* (1910) gave comprehensive descriptions of the morphology of the thrombus Until the advent of electron microscopy little has been added to this subject by later authors

Through the works of *Bizzozero* (1882) and *Eberth & Schimmelbusch* (1886 a b, and c) it was made clear that thrombosis starts with adhesion of platelets to the vascular wall followed by aggregation of additional platelets The same sequence of events was observed during the haemostatic process (*Hayem* 1882, *Lubnitzky* 1885, *Eberth & Schimmelbusch* 1886 b), with the difference that here the platelets formed a largely extravascular body, the platelet plug In previous histological studies of haemostasis (*Jorgensen & Borchgrevink* 1963 a and b 1964) the following stages in the morphological development of the platelet plugs were recognized

(1) Loosely aggregated discrete platelets with granular cytoplasm
(2) Densely packed granular platelets which often seemed to have fused although the further development indicated that this was not the case
(3) Densely packed swollen platelets with an empty or poorly stained interior The individual platelets could clearly be made out and the aggregates were surrounded by a thin fibrin membrane
(4) Apparently more loosely packed somewhat shrunken, discrete platelets surrounded by a thick fibrin membrane

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tion This was confirmed by the observation that in clotting defects, including heparin treatment the fibrin net after 24 hours was thinner and the meshes wider than normal (*Jorgensen & Borchgrevink* 1964)

In this study the morphological development of platelet aggregates and fibrin net will be reconsidered based on histological examination of experimental thrombi produced in rabbits by two different methods The intention is to elucidate certain pathogenetic mechanisms in the formation and further development of thrombi until they become organized It is felt appropriate to compare the observations in the thrombi with those in the platelet plugs and in platelet aggregates formed *in vitro* Therefore the study will include short reports on the appearance of platelet plugs in rabbits and of aggregates of human platelets formed *in vitro* through the addition to platelet rich plasma of ADP connective tissue tissue thromboplastin calcium and thrombin

An account of the reactions in the vascular wall and of the organization of thrombi will follow in a subsequent paper (*Jorgensen* 1964 a)

MATERIALS

... was ... ed with 0 9 per cent saline 1 10 or 1 25 in a few experiments 1 50 or 1 100 A fresh dilution was prepared for each experiment

Rabb ... rris Plains N.J U S A) ... its per ml (Lipo Hepin ... St Louis Mo U S A) ... icrograms per ml ... *Hovig* (1962) Dr Hovig

... *uman tissue thromboplastin* prepared from brains as described by *Owren* (1949)

Thrombin (Topostasin "Roche" Basel Switzerland) dissolved in 0 9 per cent saline to a concentration of 10 N I H units per ml

METHODS

Experim ... were u ... the r ... during

Anaesthesia was carried out by intravenous amytal sodium 0 04 g per kg body weight supplemented by a mixture of two thirds of ether and one third of 96 per cent ethan l on open mask

Thrombosis was precipitated in the femoral artery or vein by one of the following two methods

1 The vessel was caref ... d ssect on was ... d stal end of t ... ous segment ...

(5) Diffusely granular masses which take the fibrin stain throughout The outlines of the shrunken platelets were discerned with difficulty or not at all

In electron microscope the ballooning of the platelets corresponds to a reduced density of the hyaloplasm and disappearance of the organelles (*Kjærheim & Hovig* 1962), a process termed degranulation by *French & Poole* (1963)

From the changes in the haemostatic mechanism in von Willebrand's disease, *Jorgensen & Borchgrevink* (1964) found it likely that the initial adhesion to the endothelial lips is triggered by adenosine diphosphate (ADP) The subsequent platelet aggregation is precipitated by the same substance (*Gaarder, Jonsen, Laland, Hellem & Owren* 1961) or/and connective tissue fibres (*Zucker & Borrelli* 1962) According to *Hovig* (1962) the aggregates induced by ADP are looser than those formed by connective tissue "extract" Thus, the loose aggregation in the first stage may be an effect only of ADP The following stages are probably the result of a continuous formation of thrombin (*Jorgensen & Borchgrevink* 1963 a and b, 1964) However, degranulation of platelets may also be precipitated by connective tissue (*Hovig* 1962)

In larger thrombi platelet aggregates form columns or a coralline framework which extends into the vascular lumen (*Zahn* 1891, *Aschoff* 1892, *Welch* 1910) A fibrin net occupies the spaces between the aggregates, and often it forms the major part of the thrombus downstream or upstream to the platelet part Even in the formation of this net the platelets play an important rôle (*Ranvier* 1873) *Iseri & Benditt* (1961), *Parmeggiani* (1961), and *Jorgensen & Borchgrevink* (1963 a) stressed the difference in the morphological development of the platelets in larger aggregates and in the small platelet clumps entering into the formation of a fibrin net In the former case the platelets remain discrete for a long time, even after being engulfed by monocytes (*Hand & Chandler* 1962) *In the latter case the platelets disintegrate and even fuse*, also when viewed in electron microscope, forming granular bodies from which the fibrin strands radiate (*Rodman, Painter & McDevitt* 1963) *Jorgensen & Borchgrevink* (1964) suggested that the mentioned difference depends on the local concentration of thrombin In clotting blood this is higher than on the platelet surface within a large platelet aggregate Ample amounts of thrombin tend to rupture the platelet membranes (*Hovig* 1962)

In thrombi there is usually a considerable increase in the amount of fibrin with time, not only within the larger platelet aggregates but also in the fibrin net (*Aschoff* 1892) *Jorgensen & Borchgrevink* (1963 a) observed the same phenomenon *in the haematoma of the wounds made* for the primary bleeding time test 24 hours after the bleeding We considered that this was caused by a prolonged or continuous coagulation of plasma leaking out into the wound, and not only due to clot retrac-

tion This was confirmed by the observation that in clotting defects, including heparin treatment, the fibrin net after 24 hours was thinner and the meshes wider than normal (*Jorgensen & Borchgrevink* 1964).

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An account of the reactions in the vascular wall and of the organization of thrombi will follow in a subsequent paper (*Jorgensen* 1964 a).

MATERIALS

... 1 100 A fresh dilution was prepared for each experiment

Rabbit tissue thromboplastin (Simplastin Warner" Morris Plains N.J, U S A)

Heparin sodium in aqueous solution 20 000 U S P units per ml (Lipo-Hepin "Ricker", Northridge Calif, U S A)

Adenosine diphosphate dihydrate (Sigma Chemical Co, St Louis Mo, U S A) dissolved in 0 9 per cent saline to a concentration of 10 micrograms per ml

Saline extract of tendons was prepared as described by *Hovig* (1962) ...

METHODS

Exper...
were ...
their ...
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... lium, 0 04 g per kg body ...

(1) The ...

... the lumen to about ½ to ¼ of the original size ... of no 1 silk was tied, it was later checked in most cases that the vessel had not been completely occluded by this procedure (Fig 11) About 0 1–0 2 ml of diluted staphylococ

(5) Diffusely granular masses which take the fibrin stain throughout. The outlines of the shrunken platelets were discerned with difficulty or not at all.

In electron microscope the ballooning of the platelets corresponds to a reduced density of the hyaloplasm and disappearance of the organelles (*Kjærheim & Hovig* 1962), a process termed degranulation by *French & Poole* (1963).

From the changes in the haemostatic mechanism in von Willebrand's disease, *Jorgensen & Borchgrevink* (1964) found it likely that the initial adhesion to the endothelial lips is triggered by adenosine diphosphate (ADP). The subsequent platelet aggregation is precipitated by the same substance (*Gaarder, Jonsen, Laland, Hellem & Owren* 1961) or and connective tissue fibres (*Zucker & Borrelli* 1962). According to *Hovig* (1962) the aggregates induced by ADP are looser than those formed by connective tissue "extract". Thus, the loose aggregation in the first stage may be an effect only of ADP. The following stages are probably the result of a continuous formation of thrombin (*Jorgensen & Borchgrevink* 1963 a and b, 1964). However, degranulation of platelets may also be precipitated by connective tissue (*Hovig* 1962).

In larger thrombi platelet aggregates form columns or a coralline framework which extends into the vascular lumen (*Zahn* 1891, *Aschoff* 1892, *Welch* 1910). A fibrin net occupies the spaces between the aggregates, and often it forms the major part of the thrombus downstream or upstream to the platelet part. Even in the formation of this net the platelets play an important rôle (*Ranvier* 1873). *Iseri & Benditt* (1961), *Parmeggiani* (1961), and *Jorgensen & Borchgrevink* (1963 a) stressed the difference in the morphological development of the platelets in larger aggregates and in the small platelet clumps entering into the formation of a fibrin net. In the former case the platelets remain discrete for a long time, even after being engulfed by monocytes (*Hand & Chandler* 1962). In the latter case the platelets disintegrate and even fuse, also when viewed in electron microscope, forming granular bodies from which the fibrin strands radiate (*Rodman, Painter & McDevitt* 1963). *Jorgensen & Borchgrevink* (1964) suggested that the mentioned difference depends on the local concentration of thrombin. In clotting blood this is higher than on the platelet surface within a large platelet aggregate. Ample amounts of thrombin tend to rupture the platelet membranes (*Hovig* 1962).

In thrombi there is usually a considerable increase in the amount of fibrin with time, not only within the larger platelet aggregates but also in the fibrin net (*Aschoff* 1892). *Jorgensen & Borchgrevink* (1963 a) observed the same phenomenon in the haematoma of the wounds made for the primary bleeding time test 24 hours after the bleeding. We considered that this was caused by a prolonged or continuous coagulation of plasma leaking out into the wound, and not only due to clot retrac-

tion This was confirmed by the observation that in clotting defects, including heparin treatment, the fibrin net after 24 hours was thinner and the meshes wider than normal (*Jorgensen & Borchgrevink* 1964)

In this study the morphological development of platelet aggregates and fibrin net will be reconsidered based on histological examination of experimental thrombi produced in rabbits by two different methods The intention is to elucidate certain pathogenetic mechanisms in the formation and further development of thrombi until they become organized It is felt appropriate to compare the observations in the thrombi with those in the platelet plugs and in platelet aggregates formed *in vitro* Therefore, the study will include short reports on the appearance of platelet plugs in rabbits and of aggregates of human platelets formed *in vitro* through the addition to platelet rich plasma of ADP connective tissue, tissue thromboplastin, calcium and thrombin

An account of the reactions in the vascular wall and of the organization of thrombi will follow in a subsequent paper (*Jørgensen* 1964 a)

MATERIALS

Human tissue thromboplastin prepared from brains as described by *Owren* (1949)

Thrombin (Topostasin Roche Basel Switzerland) dissolved in 0 9 per cent saline to a concentration of 10 NIH units per ml

METHODS

Experimental thrombosis in rabbits Eighty eight healthy rabbits of albino breed were used Forty six were males 42 females Their median age was 21 months and their mean weight 3 173 g They were kept on a low fat standard diet prior to and during the experiment

Anaesthesia was carried out by intravenous amytal sodium 0 04 g per kg body weight supplemented by a mixture of two thirds of ether and one third of 96 per cent ethanol on open mask

Thrombosis was precipitated in the femoral artery or vein by one of the following two methods

1 The vessel was carefully exposed in about 3 cm of length No attempt at finer dissection was made and branches or tributaries were not ligated At the distal end of the exposed arterial segment or at the proximal end of the venous segment a permanent loosely applied ligature of no 1 ... constricting the l... it was later ... occluded by

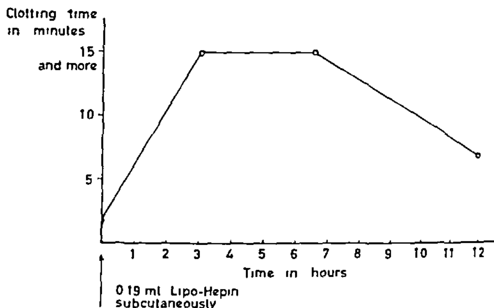


Fig 1
The clotting time in a rabbit after a single subcutaneous injection of heparin when the dose was considered satisfactory

cal α haemolysin was injected into the ventral side of the vascular wall *through several punctures with a no 20 or 22 hypodermic needle* Leakage of toxin into the gap of the wound was inevitable, but direct intravascular injection was avoided

(2) The vessel was exposed and a ligature applied as before, but now with complete occlusion of the vessel About 0 1-0 2 ml of rabbit tissue thromboplastin was injected into the vessel through a no 20 or 22 hypodermic needle Immediately afterwards the open end of the exposed segment was occluded by a bulldog clamp After 5 minutes it was removed the vessel now being filled by a coagulation thrombus which could usually be discerned through the vascular wall

When heparin treatment was given it was instituted 24 hours after the operation and continued until the end of the experiment In 2 rabbits the treatment was started half an hour after the operation The heparin was given subcutaneously every 12 hours in doses which varied from 500 to 1 150 U S P units per kg body *weight (5 11 5 mg per kg body weight) the mean dose was 816 U S P units* (8 16 mg) The dose was decided individually before the experiment by measuring the whole blod clotting time several times after test doses of heparin The clotting time was determined by filling a capillary pipette with freely flowing blood from a cut in the ear and breaking off pieces of the pipette at intervals This method was chosen because it made possible frequent determinations without destroying the ear vessels needed for the anaesthesia The clotting time was recorded as the time from the filling of the pipette until the first appearance of a fibrin thread In untreated animals this was usually 1½ 2 minutes The heparin dose was considered satisfactory when the clotting time exceeded twice the normal value 12 hours after the injection (Fig 1) The preparatory heparin treatment was discontinued for at least 48 hours prior to the precipitation of the thrombi The effectiveness of the *treatment* after the operations was checked by random tests adjustments of the dose were rarely necessary

In 11 animals one of the methods mentioned was used only on one side (Table 1) In 77 animals staphylococcal α haemolysin was injected on the left side tissue thromboplastin on the right In 22 of these heparin was given the remaining 66 animals got no further treatment The rabbits were sacrificed by decapitation from ½ hour to 50 days after the operation (Table 1) 5 rabbits died in a shocklike state

TABLE 1

Number of Rabbits Sacrificed at Various Intervals after the Operation

Treatment given	½ hour	1	2	3	5	6	7	8	10	12	17	24	[illegible]	Total
	Days													
Non heparinized														
Staphylococcal toxin left side tissue thromboplastin right side	4	7	5	2	4	1	4	1	5	4	7	7	4	55
Staphylococcal toxin left side		1	1		–		1	–	–	–	–		–	3
Tissue thromboplastin right side		–	–	2	1		1	–	1	–	1	–	2	8
Heparinized														
Staphylococcal toxin left side – tissue thromboplastin right side	–	–	5	4	4	–	3	–	2	4	–	–	–	22
Total	4	8	11	8	9	1	9	1	8	8	8	7	6	88

on the second or third day The femoral vessels with the surrounding tissue were taken out and immediately transferred to Helly's solution or formalin mercuric chloride solution for fixation

Experimental haemostasis in rabbits In 3 rabbits a cut, 1 cm long, was made at the tip of the ear with a razor blade under general anaesthesia The wounds bled freely, the bleeding times were from 6 to 10 minutes About 15 minutes after the cessation of the bleeding the cuts with the surrounding tissue were excised and transferred to Helly's solution

Human platelet aggregates formed in vitro Fifty millilitres of blood were mixed with 5 ml trisodium citrate dihydrate 3 1 per cent solution and centrifuged at 275 G for 10 minutes The platelet-rich plasma was decanted and divided into 5 portions of 2 5 or 4 ml The following additions were made·

(1) ADP, 0 3 ml (3 micrograms) to 2 5 ml plasma
(2) Saline "extract" of tendons, 0 6 ml to 2 5 ml plasma
(3) Human tissue thromboplastin, 0 4 ml of a dilution 1 100, immediately followed by $CaCl_2$ 0 4 ml 70 mM to 4 ml plasma The diluted thromboplastin time was 59 seconds
(4) $CaCl_2$, 0 4 ml 70 mM, immediately followed by ADP, 0 2 ml (2 micrograms) to 4 ml plasma
(5) Thrombin, 0 3 ml (3 N I H units) to 2 5 ml plasma

Immediately after the additions the test tubes were rotated between the palms After one minute platelet aggregation was obvious to the naked eye The aggregates were centrifuged at 125 G for 10 minutes, the supernatant plasma or serum *decanted* and Helly's solution added

Histological technique Among several fixation fluids tried, Helly's solution gave the best results, particularly in fresh thrombi After 24 hours of fixation the specimens were rinsed in flowing tap water for another 24 hours With older thrombi nearly equal results were obtained by the formalin mercuric chloride method of *Lendrum Fraser, Slidders & Henderson* (1962), combined with fixation for 7 days in 5 per cent aqueous mercuric chloride

After fixation the femoral vessels were cut transversely in blocks 0 3 0 4 cm long embedded in paraffin and cut in transverse or longitudinal sections about 5 microns thick at several depths The following stains were used

(1) Masson's haematoxylin erythrosin saffron
(2) The acid picro Mallory method for staining fibrin (*Lendrum* 1949)
(3) The martius scarlet blue (MSB) method of *Lendrum Fraser Slidders & Henderson* (1962)
(4) Mallory s phosphotungstic acid haematoxylin (PTAH) method

RESULTS

In this paper the word thrombus comprises every intravascular body derived from blood constituents, including the smallest platelet aggregate provided it was connected with the vascular wall The staphylococcal α-haemolysin gave platelet thrombi or mixed platelet and coagulation thrombi the tissue thromboplastin gave coagulation thrombi The number of thrombi obtained at different periods of time after the operation is shown in Tables 2 and 7 With staphylococcal toxin non intentional thrombi often arose in the vessel adjacent to the treated one The number of these is indicated in brackets, and they are included in the material Unless otherwise stated, there was no difference between arterial and venous thrombi produced by the same method

TABLE 2

Number of Thrombi Observed at Various Intervals after the Operation in 66 Non Heparinized Rabbits

Type of thrombi	½ hour 1 day		2-3 days		5-6 days		7-8 days		10-12 days		17 days		28 days		50 days		Total
	Art	Ven	Art	Ven	Art	Ven	Art	Ven	Art	Ven	Art	Ven	Art	Ven	Art	Ven	
Staphylococcal toxin																	
Thrombi with platelets fibrin	5(3)	7(4)	2(4)	6(2)	1(2)	4	2(2)	4(1)	2(3)	4	(2)	2	1(1)	1	(1)	2	43(25)
Completely organized thrombi				–		–			2	1(2)	3	1	3(2)	2	2(1)	(1)	14(6)
No thrombi												1					1
Total	5(3)	7(4)	2(4)	6(2)	1(2)	4	2(2)	4(1)	4(3)	5(2)	3(2)	4	4(3)	3	2(2)	2(1)	58(31)
Tissue thromboplastin																	
Thrombi with fibrin	4	7	4	5	1	4	3	3	5	1	1			–			38
Completely organized thrombi	–		–		–	–		–	–	3	3	4	4	3	3	2	22
No thrombi	–	–			1	–		–	–	1				–		1	3
Total	4	7	4	5	2	4	3	3	5	5	4	4	4	3	3	3	63

Figures in brackets indicate non intentional thrombi in the vessel adjacent to the treated one

on the second or third day The femoral vessels with the surrounding tissue were taken out and immediately transferred to Helly s solution or formalin mercuric chloride solution for fixation

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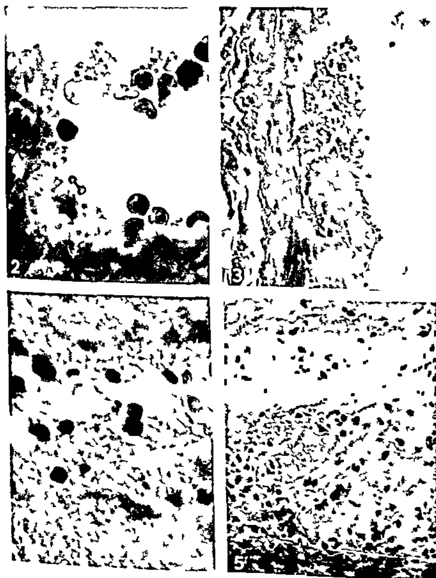
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diameter up to 2 3 microns (Fig 4) Usually the last mentioned aggregates were equipped with a more or less distinct fibrin membrane at the surface or near and parallel to it In aggregates with the membrane buried at a certain depth the platelets lying outside tended to be more swollen than those inside (Fig 4) Beginning at 24 hours a fourth stage in the development of the aggregates was observed more or less compact masses of dense small platelets with a diameter of less than 1 2



Figs 2 5

TABLE 3
Types of Thrombi Produced by Staphylococcal α Haemolysin in Non Heparinized Rabbits

Type of thrombi	½ hour		1–3 days		3–4 days		8 days	
	Art	Ven	Art	Ven	Art	Ven	Art	Ven
Loose aggregates connected with the wall	2	2	–	1		1		1
Non occluding mainly platelet trombi in a more advanced stage	–	–	5	2	1	2	–	–
Occluding mixed thrombi		1	7	13	2	1	4	4
Total	2	3	12	16	3	4	4	5

Intentional and non intentional thrombi are pooled

Platelet Thrombi and Platelet Part of Mixed Thrombi Made by Staphylococcal α-Haemolysin in Non-Heparinized Rabbits

Invariably, injection of staphylococcal toxin resulted in coagulation necrosis of the vascular wall and the surroundings, often including the neighbouring vessel After the first days a zone of leucocytes formed *a characteristic band around the necrotic area The thrombi varied* considerably and were observed in all stages of their development A common feature of unorganized thrombi was the presence of platelet *aggregates In 7 instances only small, loose clumps of granular plate*lets occurred, loosely connected with the vascular wall (Fig 2) (Table 3) In several other vessels larger aggregates were well fixed to the wall They were either composed of *densely packed platelets*, often apparently fused (Fig 3), or of slightly swollen, degranulated platelets with a

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Figs 2 5

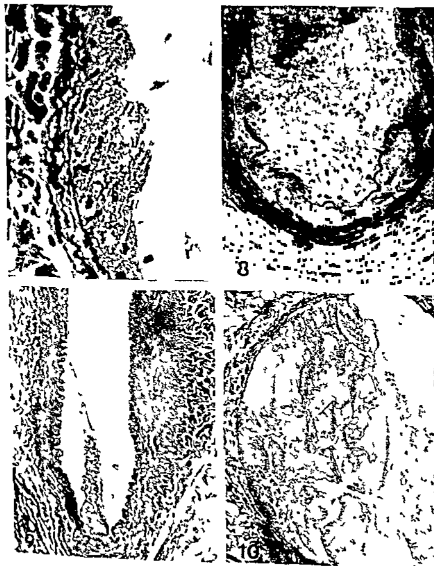
Fig 2 A few loosely aggregated granular platelets adhere to the vascular lining *At the top a small detached platelet clump Femoral vein beside artery* treated with staphylococcal toxin ½ hour after the operation Haematoxylin erythrosin saffron × 900

Fig 3 Platelet thrombus with densely packed granular platelets At upper right a small detached platelet clump Femoral vein treated with staphylococcal toxin 24 hours after the operation Lendrum's stain × 260

Fig 4 Part of a coralline platelet thrombus with slightly swollen and degranulated platelets and peripheral fibrin membrane The platelets are more swollen outside the fibrin membranes (in the middle) than inside (at the top and bottom) *Femoral vein treated with staphylococcal toxin 24* hours after the operation Lendrum's stain × 800

Fig 5 Part of a coralline thrombus with compact masses of small granular platelets interspersed with fibrin net Femoral artery beside vein treated *with staphylococcal toxin 2 days after the operation Haematoxylin erythro*sin saffron × 200

downstream to the stenosing ligature (Fig 11) The large aggregates had at times the shape of perpendicular platelet columns spaced out at intervals (Fig 12) These columns were twisted, bended, and branched, occasionally thrown into an irregular pile The classic coralline structure appeared when the columns were connected with bars of platelet masses parallel to the wall The perpendicular columns tended to widen towards the open lumen, and at the luminal surface the platelet throm



Figs 7 10

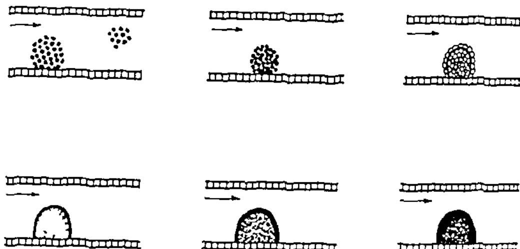


Fig 6
Diagram summarizing the morphological changes of platelet thrombi

microns (Fig 5) These aggregates differed from the early ones by their larger size and their well-defined perimetric fibrin membrane From the same time several of the aggregates were in various stages of fibrinous transformation the fibrin membrane broadened, and fibrin strands precipitated between the platelets (Fig 7) As the amount of fibrin increased, the platelets were concealed, resulting in a fibrin mass with a granular appearance However, aggregates in the middle of mixed thrombi often failed to go through the fibrinous transformation, even after 50 days (Fig 8) The sequence of changes of the aggregates is summarized in Fig 6 A variable amount of white and red blood cells, particularly granulocytes, was seen in the aggregates at all stages

The small and medium-sized aggregates were often flat, clinging to the vascular wall (Figs 3 and 7) Occasionally, they were erected perpendicular to it (Fig 9), or formed whorl-like structures, particularly

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Figs 7–10

Fig 7 Platelet thrombus in which fibrin has precipitated nearly concealing all the platelets Femoral vein treated with staphylococcal toxin 2 days after the operation MSB × 300

Fig 8 Mixed thrombus with a peripheral zone of compact aggregates of small platelets outlined by a distinct wavy fibrin membrane The delicate net partly lysed A few shadows of erythrocytes remain Femoral artery beside vein treated with staphylococcal toxin 50 days after the operation PTAH × 120

Fig 9 Platelet column perpendicular to the wall A fibrin membrane from the tip of the column to the wall probably heralds the precipitation of a coagulation part Femoral artery beside vein treated with staphylococcal toxin 24 hours after the operation Lendrum's stain × 25

Fig 10 Coralline platelet thrombus with widening columns and bars towards the open lumen At the bottom a fragment is on the verge of breaking off Femoral vein treated with staphylococcal toxin 24 hours after the operation MSB × 25

bus often terminated in a particularly thick bar (Fig 10) Usually two or more of the mentioned developmental stages were represented in the same aggregate and the youngest parts were always located nearest the lumen Here fragments of the aggregates were commonly on the verge of breaking off from non occluding thrombi (Fig 10) and detached masses could be seen in open lumen (Figs 2 and 3) This which is indicative of an active thrombotic process was not only observed in the first days but even in 1 arterial thrombus after 28 days and in 7 venous thrombi after 5 to 50 days (Fig 13) One artery observed after 17 days deserves special mention Here a defect in the media had prompted the precipitation of dense granular crumbling aggregates without fibrin (Fig 14)

The platelet thrombi were located on both sides of the stenosing ligature but the favourite sites were downstream to it and just at the stenosis (Fig 11) Platelet massing was likewise apt to occur behind venous leaflets

As to the size of the aggregates Table 4 indicates a difference between arterial and venous thrombi It seems to be a tendency for platelet aggregates to develop more slowly in arteries than in veins Thus there was no large aggregate in arteries earlier than after 2 days while 5 of 8 aggregates in veins after 24 hours were of this type All 3 large arterial aggregates after 2 and 3 days were non intentional whereas there was no trend in the distribution of venous aggregates according to size when the figures are split into intentional and non intentional thrombi The largest aggregates were found in non intentional arterial thrombi after the 2nd day

Four vessels treated with staphylococcal toxin and 17 vessels treated with tissue thromboplastin all with restored circulation via collaterals or past old endothelialized lesions contained freely floating platelet aggregates the forming site of which could not be determined (Figs 15 and 16) Usually they were small and loose occasionally larger dense and granular without fibrin In a few instances they had coalesced into conglomerates of discrete or connected granular aggregates with a vague fibrin membrane and a fringe of ballooned platelets (Fig 17)

Coagulation Part of Mixed Thrombi Made by Staphylococcal α Haemolysin and Coagulation Thrombi Made by Tissue Thromboplastin in Non Heparinized Rabbits

The first sign of clotting was the precipitation of fibrin membranes fixed to platelet thrombi or the vascular wall (Fig 9) In the completed coagulation thrombus the membranes formed a coarse framework Figs 11 and 18) This included a peripheral membrane and in the mixed thrombi one or more parallel membranes within the thrombus running in a streamline pattern In both types of thrombi arched and wavy membranes branched off from the peripheral one or from plate





Figs 11 19

Fig 11 M xe l thr mb us on either side f the sten ing ligat ir Fl w from left t r ght Darl er grey areas at the stenosis an l d wn tream to it are platelet parts Streamline pattern of fibrin membran n the coagula ti part Femoral ve n treatel w th tap l y l ccal t x n 2 days after the perati n Len lrum s stain × 25

Fig 12 M xe l thr mb us with parall l c lumns spa e l ut at intervals Flow fr m l ft t right Fem ral vein treated with staph yloc al t xin 6 l fter the operat on Lendr im s stain × 25

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Figs 11 12

Fig 11 Mixed thrombus on either side of the stenosing ligature Flow from left to right Darker grey areas at the stenosis and downstream to it are platelet parts Streamline pattern of fibrin membranes in the coagulation part Femoral vein treated with staphylococcal toxin 2 days after the operation Lendrum's stain × 25

Fig 12 Mixed thrombus with parallel columns spaced out at intervals Flow from left to right Femoral vein treated with staphylococcal toxin 6 days after the operation Lendrum's stain × 25

few platelets, and measured 5–15 microns in the freshest thrombi Larger aggregates with granular or slightly degranulated platelets were intimately connected with the fibrin membranes, not rarely squeezed between two adjacent ones (Fig 19) At the upstream end of thrombi, and at the mouths of venous tributaries, aggregates of the same type as those described in the preceding paragraph were encountered on the surface of the thrombi

TABLE 4

Occurrence of Small or Medium Sized vs Large Platelet Thrombi or Platelet Part of Mixed Thrombi Produced by Staphylococcal α Haemolysin

Type of platelet aggregates	Arterial thrombi			Venous thrombi		
	½ hour	1 day	2 3 days	½ hour	1 day	2 3 days
Small and medium sized	2	6	3	3	3	6
Large	0	0	3	0	5	2
Total	2	6	6	3	8	8

Intentional and non intentional thrombi are pooled
Fisher exact probability test (½ hour and 1 day grouped together)

Arterial thrombi P = 0 11 (two tailed)
Venous thrombi P = 0 67 (two tailed)

As the platelet thrombus, the coagulation variety undergoes profound changes with time In the toxin produced thrombi the erythrocytes were mostly haemolyzed already after 24 hours, while in the thromboplastin made thrombi this was the case only to a slight extent even in the oldest unorganized remnants From the 2nd day the leucocytes showed elongation of the nuclei, pycnosis and karyorrhexis Any open space around the thrombus widened The amount of fibrin increased steadily until fibrinolysis supervened, particularly corresponding to platelet rich parts of the coagulation thrombus The platelet aggregates along the membranes underwent fibrinous transformation

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Figs 13 16

Fig 13 Active platelet thrombus on a thickened vascular wall Femoral vein treated with staphylococcal toxin 17 days after the operation *Lendrum s stain* × 105

Fig 14 *Crumbling* very dense granular platelet thrombus in a defect of the media accidentally caused during the operation Femoral artery beside vein treated with staphylococcal toxin 17 *days after* the operation Lendrum s stain × 105

Fig [illegible] [illegible] [illegible]ylococcal [illegible] days after the operation Lendrum s stain × 105

Fig 16 Freely *floating* conglomerate of granular aggregates with a fringe of ballooned platelets Femoral artery treated with staphylococcal toxin 12 days after the operation Heparinized *animal* MSB × 22

let aggregates in the mixed type Frequently, they lined channels within the thrombus, filled by unclotted blood However, most of the spaces between the membranes were filled by a delicate fibrin net in which red and white blood cells were entrapped, the latter particularly along the membranes, including those of the platelet thrombi In some of the intersections of the net finely granular bodies appeared as knots and stained as fibrin (Fig 19) They were probably derived from one or a









Figs 13 16

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Fig 15 Freely floating aggre[illegible] site caught in a na[illegible] flow from left Fe[illegible] toxin 7 days after t[illegible]

Fig 16 Freely floating conglomerate of granular aggregates with a fringe of ballooned platelets Femoral artery treated with staphylococcal toxin 12 days after the operation Heparinized animal MSB × 22

and the knots of platelet origin tended to enlarge and fuse Especially in the mixed thrombi also the membranes and the delicate net became coarser The red blood cells, haemolyzed or not, were strangulated and finally concealed by the fibrin net. At this stage the fibrin appeared as broad hyaline bands or masses with small clear spaces in which elongated leucocyte nuclei lingered for a while (Fig 20). At this stage fibrin which had buried the coagulation part of the mixed thrombi could not



Figs 17 20

be distinguished from the fibrin which concealed the platelet part

The increase of fibrin was most marked at the upstream end of the thrombi and in the pure coagulation thrombi, at the injection site In the middle of the occluding mixed thrombi the increase was only slight, and here *the stroma of laked erythrocytes* shrank and disappeared, causing collaps of the fibrin net

In the toxin produced thrombi complete occlusion of the vessel was effected by the coagulation part, and not by the platelet part alone However, the proportion of the two components varied considerably Even in a few non intentional arterial thrombi the platelet aggregation part was rather small As seen in Table 5 all 6 non-intentional venous thrombi observed after 1–8 days were occluding, while this was the case in only 5 of 11 non intentional arterial thrombi This is a reflection of the fact that precipitation of the coagulation part takes place more readily in veins than in arteries Within either category of vessel the relative frequency of occluding and non occluding thrombi varied at the different points of time, but it did not show any definite trend after the first day (Table 3) The concentration of the injected toxin did not seem to influence this ratio

TABLE 5

Non Occluding vs Occluding Non Intentional Arterial and Venous Thrombi Produced by Staphylococcal α Haemolysin Observed between 1 and 8 Days after the Operation

Type of thrombi	Arterial	Venous
Non Occluding	6	0
Occluding	5	6
Total	11	6

Fisher exact probability test P = 0 075 (two tailed)

Figs 17 20

Fig 17 Detail of Fig 16 MSB × 260

Fig 18 Coagulation part of mixed thrombus with membranous framework including a peripheral membrane Femoral vein treated with *staphylococcal* toxin 2 days after the operation Lendrum s stain × 13

Fig 19 Coagulation thrombus with platelet *aggregates* betw[illegible]

Fig 20 [illegible] with hyaline fibrin masses near the upstream end [illegible] f the clear spaces contain a leucocyte in degeneration Femoral vein beside artery treated with staphylococcal toxin 7 days after the operation Lendrum s stain × 105

TABLE 6

Signs of Spontaneous General Fibrinolysis in Mixed Thrombi Made by Staphylococcal α Haemolysin and in Coagulation Thrombi Made by Tissue Thromboplastin

Type of thrombi	½ hour 1 day		2-3 days		5-6 days		7-8 days		10-12 days	
	Art	Ven	Art	Ven	Art	Ven	Art	Ven	Art	Ven
	Staphylococcal toxin									
Without signs of fibrinolysis	3	7	4	7	2	–	1	2	5	7
With signs of fibrinolysis	–	–	–	–	–	1	3	2	2	–
	Tissue thromboplastin									
Without signs of fibrinolysis	4	7	4	4	–	–	2	1	1	4
With signs of fibrinolysis			–	1	1	4	1	2	4	–
No thrombi			–		1	–	–	–	–	1

Intentional and non intentional mixed thrombi are pooled

TABLE 7

N mber f Thron b Obser e l at Var o s Inter ls after the Operat on n 22 Hepar n ze l Rabb ts

T p f t o	1 n		la s		7 ays		10-12 ays		T l
	A	V	A	Ve	Art	V	Ar	V n	
St phyl c ccal tox n									
t romb ith platel ts f b rin	3(2	4(1)		1	1(1	1	2	1(1)	15(5)
mpl el rga ize l thr mb							1	1	2
th r mb	1	1		1		1		1	5
tal	4(2	5(1)	2		1(1)	2	3	3(1)	2?(5)
T ss e thro l oplast									
hr mb i ith f b rin	4	5	1	2	1	1	2		1?
ompl tely rganiz l thromb i						1	1	3	5
thr mb i			1						1
tal	4	5	2		1	2	3	3	22

I ig es n l rack ts i l cate no i intentional thromb i in th e e sel a ljacent to th e treate l one
In 2 rab b ts k illed after 2 an l 7 lays th e h parin treatment as starte l ½ h r after th e operati n

TABLE 6

Signs of Spontaneous General Fibrinolysis in Mixed Thrombi Made by Staphylococcal α-Haemolysin and in Coagulation Thrombi Made by Tissue Thromboplastin

Type of thrombi	½ hour 1 day		2-3 days		5-6 days		7-8 days		10-12 days	
	Art	Ven	Art	Ven	Art	Ven	Art	Ven	Art	Ven
	Staphylococcal toxin									
Without signs of fibrinolysis	3	7	4	7	2	–	1	2	5	7
With signs of fibrinolysis	–	–		–	–	1	3	2	2	–
	Tissue thromboplastin									
Without signs of fibrinolysis	4	7	4	4	–	–	2	1	1	4
With signs of fibrinolysis			–	1	1	4	1	2	4	–
No thrombi		–	–	–	1	–	–	–	–	1

Intentional and non intentional mixed thrombi are pooled

5th day in the thromboplastin made thrombi It was more pronounced than usual particularly in venous—and thromboplastin made—thrombi Among unorganized thrombi only 2 of the mixed type and 2 of the coagulation type lacked any sign of fibrinolysis

As in non heparinized animals vessels with restored circulation contained young freely floating platelet aggregates or conglomerates of such aggregates (Figs 16 and 17) However, they were more frequent in heparinized animals 11 vessels treated with staphylococcal toxin and 10 treated with tissue thromboplastin showed these aggregates *i e*, nearly half of the vessels As stated above, the corresponding figures in non heparinized animals were 4 and 17, *i e*, about one sixth of the vessels But this comparison is not quite fair because the aggregates in the heparinized animals were seen even in the first days, at a time when in non heparinized animals the circulation was often completely blocked by unlysed thrombi Nevertheless within each vessel of the heparinized animals the aggregates were more numerous and larger than usual

Platelet Plugs in Rabbits

Platelet plugs were not only observed in the ear wounds of the 3 rabbits but also at the puncture site in several of the vessels treated with tissue thromboplastin In most respects they were identical to those observed in man (*Jorgensen & Borchgrevink* 1963 a and b) However, their morphological development was slower Fifteen minutes after the bleeding the plugs had often not reached the stage of ballooning and those who had showed less swelling of the platelets than many human plugs at the same time (Fig 24) The marked ballooning of the platelets was obtained in some of the puncture site plugs at 24 hours (Fig 25) The fibrinous transformation was completed after 2 days, sometimes after 24 hours

Human Platelet Aggregates Formed in vitro

ADP precipitated small loose collections of granular platelets (Fig 26) Morphologically they were indistinguishable from (1) those observed as the first stage of platelet thrombi (2) many of the young freely floating aggregates the origin of which could not be traced, and (3) the platelet aggregates covering the mouths of the bleeding vessels in wounds with severe clotting defects (*Jorgensen & Borchgrevink* 1964)

Saline extract of tendons gave at the same point of time larger, irregularly formed aggregates with varying density (Fig 27) Platelets were closely clumped together often obscuring the outlines of the individual platelet In between platelets were more loosely arranged Their cytoplasm was granular and dense with the exception of a few scattered degranulated platelets No fibrin was present The picture had

stream (Fig 8) In general, the fibrinolysis was more marked in veins than in arteries, and more marked in the thromboplastin-made thrombi than in the toxin-produced A consequence of this is the fact that arterial thrombi, and toxin-produced, healed with more extensive permanent sequelae than their respective counterparts (*Jorgensen* 1964 a)

Heparin Treatment

Heparin medication after the initiation of thrombosis by staphylococcal toxin reduced the number of vessels with thrombi from 57 of 58 (98 per cent) to 17 of 22 (77 per cent) (Tables 2 and 7) Further, the number of non-intentional thrombi decreased from 31 in 58 non injected vessels (53 per cent) to 5 in 22 (23 per cent) These differences are below the conventional level of significance when tested by the χ^2 method ($P < 0\ 01$ and $0\ 05 > P > 0\ 02$ respectively) There was no difference in the occurrence of coagulation thrombi produced by tissue thromboplastin whether heparin was given or not

TABLE 8
Signs of General Fibrinolysis under Heparin Treatment in Mixed Thrombi Made by Staphylococcal α Haemolysin and in Coagulation Thrombi Made by Tissue Thromboplastin

Type of thrombi	2-3 days		5 days		7 days		10-12 days	
	Art	Ven	Art	Ven	Art	Ven	Art	Ven
	Staphylococcal toxin							
Without signs of fibrinolysis		–	1			1	1	2
With signs of fibrinolysis	4	4	1	1	2		2	1
No thrombi	1	1		1		1	–	1
	Tissue thromboplastin							
Without signs of fibrinolysis	–	2	–	1	–	1	1	3
With signs of fibrinolysis	4	3	1	1	1	1	2	
No thrombi			1	–			–	

Intentional and non intentional mixed thrombi are pooled

Two of the heparinized toxin-produced thrombi after 2 and 3 days consisted only of small loose platelet aggregates connected with the wall The remaining thrombi made by this method were mixed thrombi The fibrin amount of most thrombi was not or only slightly less than that of the non-heparinized Only 2 mixed and 2 coagulation thrombi in 4 different rabbits killed after 5 days or earlier had obviously a more delicate and wide meshed *fibrin net than usual Much more conspicuous* was the tendency towards general fibrinolysis (Fig 22) (Table 8) It started already after 2 days and, due to progressing organization, subsided from the 10th day in the toxin produced thrombi, from the

5th day in the thromboplastin made thrombi It was more pronounced than usual particularly in venous—and thromboplastin made—thrombi Among unorganized thrombi only 2 of the mixed type and 2 of the coagulation type lacked any sign of fibrinolysis

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Figs 24–29

Fig 24 Plug with slightly ballooned platelets in front of a capillary vein. Ear wound in a rabbit 15 minutes after the bleeding. MSB × 240

Fig 25 Plugs with more marked ballooning of the platelets at the puncture site of a vein treated with tissue thromboplastin 24 hours after the operation. Lendrum × 240

Fig 26 Loosely collected granular platelets. Produced by [illegible] phosphate. Haematoxylin erythrosin saffron × 800

Fig 27 Platelet aggregate of varying density; many platelets [illegible] clumped together. Produced by saline extract [illegible]. PTAH × 3 0

Fig 28 Platelet aggregate with a granular centre and a fringe of ballooned platelets outside a faintly indicated fibrin membrane. Produced by tissue thromboplastin. MSB × 390

Fig 29 Densely packed platelet aggregate in a delicate net of fibrin with several black knots of platelet origin. Produced by thrombin. PTAH × 390

no great likeness to any aggregate observed in experimental thrombi or haemostasis perhaps except the thrombus shown in Fig 14

Tissue thromboplastin and recalcification plus ADP gave dense granular aggregates with a faintly indicated fibrin membrane near and parallel to the surface (Fig 28) Platelets lying outside this membrane were clearly swollen and degranulated The thrombin induced aggregates were similar but they lacked the characteristic fibrin membrane and the ballooned platelets (Fig 29) The former type of aggregates were identical to those of the freely floating conglomerates whereas the latter type resembled the granular aggregates connected with the fibrin membranes of coagulation thrombi By all three methods a young delicate fibrin net had precipitated containing knots of platelet origin smaller than the granular bodies of the coagulation thrombi (Fig 29)

Aggregates with ballooning of platelets throughout as in naturally occurring platelet plugs and thrombi could not be precipitated by any of the methods

DISCUSSION

In the pathogenesis of thrombosis three sets of predisposing factors have repeatedly been discussed viz. lesions of the vascular wall mechanical disturbances of the circulation and alteration of the blood (*Virchow* 1856 *Welch* 1910 *Aschoff* 1938 *Poole & French* 1961 *Mustard Murphy Rowsell & Downie* 1962) In this experimental study these factors have been combined in two different ways By injection of staphylococcal toxin and partial ligation all three factors became involved in so far as vascular necrosis slowing and eddying of the blood flow and local hypercoagulability were created By injection of tissue thromboplastin and occluding ligation mainly alterations in the circulation and blood were produced Of course the pathogenesis of the thrombi formed may be different from most naturally occurring thrombi in man and the present observations cannot be generally applied without reservations

The preparation of staphylococcal toxin contained fairly large amounts of α-haemolysin as opposed to other haemolysins (*Omland* 19[illegible]) Other staphylococcal toxins are assumed to be present but this has not been tested (*Omland* 1964) The presence of necrotizing dermotoxin however is evident from the wide spread necrosis identical to that observed in human infections (*Larsen & Jorgensen* 1960) Production of coagulase is usually associated with that of α haemolysin (*Elek & Levy* 19[illegible]) Nevertheless with the severe vascular lesions created here it is likely that a significant amount of thrombin is also generated via usual mechanisms *i e* the extrinsic clotting system triggered by tissue thromboplastin and the intrinsic clotting system whether this is started by activation of Hageman factor (Factor XII) tissue thromboplastin (*Waaler* 1957) or extracellular tissue fluid (*Ouren* 1962)

In spite of this, the initial step in the toxin-produced thrombosis in flowing blood was not fibrin precipitation, but platelet adhesion and aggregation In accordance with the electronmicroscopical findings of *Poole, French & Cliff* (1963) the earliest platelet thrombi consisted of loose aggregates of granular platelets They were morphologically identical to the platelet plugs in wounds where the clotting was most impaired (*Jorgensen & Borchgrevink* 1964) The same type of aggregates was produced *in vitro* by the addition of ADP to platelet-rich plasma confirming the electronmicroscopical observations by *Hovig* (1962) Likewise in conformity with *Hovig* (1962), aggregates formed by tendon "extract" were, by the same time, generally more dense But the density varied, which gave the masses a clumpy appearance, unlike most platelet aggregates in thrombosis or haemostasis These findings are compatible with the concept that it is ADP, and not connective tissue, which brings about the initial aggregation If so, it is also likely that the initial adhesion of the platelets to the damaged wall is precipitated by ADP, as previously indicated in the case of human haemostasis (*Jorgensen & Borchgrevink* 1964) *Honour & Mitchell* (1963) demonstrated *in vivo* that application of ADP to the vascular wall prompted platelet thrombosis but, in higher concentrations, ATP and 5-hydroxytryptamine had the same effect When collagen fibres are directly exposed, as in the defective artery with platelet thrombus at 17 days, depicted in Fig 14, the connective tissue factor may be important in the initiation of the thrombosis

On the whole, the development of the platelet thrombus through the stages of apparent fusion and ballooning was the same as in the platelet plug (*Jorgensen & Borchgrevink* 1963 a and b) The ballooning of the platelets did not reach the same degree as in some of the human platelet plugs, but here interspecies differences must be considered The morphological development of the platelet plugs in rabbits were slower than in man Nevertheless, the thrombotic aggregates had certain features in common with human plugs formed under defective coagulation (*Jorgensen & Borchgrevink* 1964) There was obviously a prolonged phase of fragmentation, embolization, and rebuilding of many thrombi The reason for this may be that in the swift flow platelet aggregates are immediately torn away before enough thrombin has had time to form on the platelet surface to stabilize the aggregate

Fragmentation, embolization, and rebuilding of fresh platelet thrombi have been described by all *in vivo* observers of thrombosis, first by *Jones* (1851) However, most of these studies have been based on short time experiments, lasting hours at the most In the present investigation evidence is presented that in rabbits this may go on for several weeks A clinical counterpart in man is probably the repeated transient attacks of monocular visual loss caused by platelet emboli in the retinal vessels in cases of mural carotid thrombosis (*Russell* 1961, *McBrien*,

Bradley & Ashton 1963) Situations analogous to this may well be common even in other vascular beds

Even though the precipitation and further development of platelet thrombi are dependent on humoral factors as ADP and thrombin the significance of haemodynamic factors must not be underrated (*Eberth & Schimmelbusch* 1886 a *Aschoff* 1913 *Murphy Rowsell Downie Robinson & Mustard* 1962) They are probably decisive for the exact localization of the platelet thrombi Otherwise their particular occurrence at sites of eddying could not be explained *Rowsell Geissinger & Mustard* (1964) found platelet aggregation especially corresponding to sites of changed flow pattern when a diffuse endothelial injury was produced In our experiments platelet massing was preferentially located at and downstream to the stenosis in accordance with the experience from flow chambers (*Shionoya* 1927 *Mustard Murphy Rowsell & Downie* 1962) and vascular grafts (*Eiken* 1961) Further the regular spacing of the platelet columns and the coralline structure can hardly be accounted for without ascribing a great importance to the presence of eddies The increasing volume of coralline aggregates towards the open lumen may be due to increasing turbulence of flow as the thrombus is encroaching the lumen

Two circumstances should predispose to a more rapid accumulation of platelets in arteries than in veins More platelets per unit of time are carried to the site of thombosis and turbulence is more likely to arise at a local restriction of the lumen due to the more rapid flow On the other hand there is no doubt that platelet thrombosis is hampered by the crumbling effect of the swift arterial flow In the present experiments there was a certain but not statistically significant trend for arterial platelet thrombi to grow more slowly than venous However at 2 days and later large aggregates prevailed in the non intentional arterial thrombi Less than half of these at 1–8 days was occluding while this was the case in all non intentional venous thrombi by the addition of a coagulation part This of course causes a break in the further growth of the original platelet trombus The delay in the precipitation of the coagulation part in many non intentional arterial thrombi gave the platelet thrombus time to reach a considerable size Thus the often stressed factor of retarded blood flow appears to be of minor importance to platelet thrombi *per se* when the local stimulus to their formation is strong and enduring

On the other hand in the genesis of coagulation thrombi hindrance to the blood flow plays a major role (*Zahn* 1875 *Eberth & Schimmelbush* 1886 c) It has been considered unlikely that thrombin could form via the intrinsic clotting system unless there is almost complete stagnation of the blood (*French* 1962) *Wessler* (1955) produced coagulation thrombi at occluding ligatures by infusion of serum containing a clot promoting substance while ligation or serum infusion alone did not have this effect However already *Baumgarten* (1886) maintained

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Platelets were found in connection with coagulation thrombi in 3 situations (1) as knots in the delicate net (2) as larger aggregates adhering to the coarse membranes (3) as secondary platelet thrombi on the free surface Already at ½ hour the knots of the net were larger than those found in the recently formed *in vitro* clots and in the haema toma of the test wounds shortly after the bleeding (*Jorgensen & Borch grevink* 1963 a) According to *Benthaus & Grunberg* (1958) this is due to retraction of the fibrin net The larger aggregates were similar to those produced *in vitro* by thrombin except that some of the former had slightly degranulated platelets not matched in the latter However in the electron microscope thrombin induced aggregates do show a varying degree of degranulation (*Hovig* 1962) although the platelets are not swollen and their cytoplasm not rarified to such a degree that one should expect to recognize the changes as ballooning in the light microscope The close contact of the larger aggregates with the fibrin membranes makes it likely that even they are formed in the clotting milieu at the surface between two laminae of flow Secondary platelet thrombi on the free surface of coagulation thrombi have been known since *Aschoff* (1892) and they may be explained by the great affinity of platelets to fibrin (*Schimmelbusch* 1885) and exudation of clot promoting serum from the retracting coagulation thrombus (*Quick* 1950)

As expected the amount of fibrin in the thrombi increased with time often up to a point where any original structure was concealed by hyaline masses of fibrin The platelet thrombi underwent fibrinous transformation similar to that found in the platelet plug (*Jorgensen & Borchgrevink* 1963 a) but the intermediate stage of shrunken plate lets and thick perimetric membrane was persistent in several instances especially in the middle of mixed thrombi This and the fact that the coarse fibrin masses were particularly found at the upstream end of the thrombi point to the importance of an infiltration of plasma into the thrombus The role played by local activation of the clotting is prob ably illustrated by the tendency of the fibrin to precipitate in associa tion with any collection of platelets—or at the damaged wall at the injection site in the primary coagulation thrombi The poor effect of heparin in preventing the increase of fibrin may partly be a conse quence of the neutralizing action of platelet factor 4

The observation of fibrinous transformation of platelet aggregates may form a bridge *between the two schools of adherents* to the en crustation theory in the genesis of atherosclerosis *viz* those who consider fibrin precipitation as the primary event (*Duguid* 1946 *Crawford & Levene* 1952 *Astrup* 1959) and those who stress the import ance of platelet thrombi in this connection (*Hand & Chandler* 1962 *Murphy Rousell Downie Robinson & Mustard* 1962)

The increase of fibrin was succeeded by fibrinolysis both in a gene ral and local form The morphological criteria of general fibrinolysis

that complete arrest of flow is not a prerequisite for the formation of a coagulation thrombus, and *Wessler* (1955) showed that only partial ligation combined with serum infusion gave coagulation thrombi, although less frequently. *Shionoya* (1927) observed directly in his extracorporeal shunt that the clotting started while blood still was flowing. In the present study complete occlusion of the vessels was not effected by platelet thrombi alone, but by precipitation of a coagulation part, more readily in veins than in arteries when the issue was not complicated by the stenosing ligature. The coagulation thrombi had a framework of fibrin membranes often running in streamline patterns, and they were the first structures to be laid down. Most likely, these membranes are formed at the contact surface between two laminae of flow, conceivably promoted by exchange of, and a steady new supply of activated clotting factors at this level. The frequent connection of the membranes with the platelet thrombi suggests release of clotting activity from these structures. In the coagulation thrombi upstream to the occluding ligature the presence of the coarse fibrin framework is consistent with a slow eddying movement of blood during their development and growth. Similar fibrin membranes, appearing as ring figures, were observed by *Jorgensen & Borchgrevink* (1964) in the haematoma of test wounds in patients with prolonged bleeding time, whereas they were not found in clots formed in completely non-moving blood (*Jorgensen* 1964 b). Here only a delicate net was observed, similar to that which was secondarily precipitated within the framework of the thrombi. All this evidence supports the concept that coagulation thrombi are formed in retarded, but still flowing blood, and that the occurrence of well-defined fibrin membranes in a coagulation thrombus is a strong indication of its intravital origin. These membranes were mentioned by *Zahn* (1875) and *Aschoff* (1892), but later authors have usually found that coagulation thrombi do not differ from clots (*Welch* 1910, *Wessler, Reiner, Freiman, Reimer & Lertzman* 1959, *French* 1962).

After this, one should expect a primary coagulation thrombus to form when the vessel is treated with staphylococcal toxin and partial ligation, creating both hypercoagulability and retardation of flow. When this did not occur, it may be explained by the fact that platelet adhesion and aggregation takes place more rapidly than intravascular clotting. Admittedly, in some instances the toxin and the ligature may have been of greater importance to the coagulation part than the modest amount of platelets which had gathered before the clotting. However, it may be difficult to reconstruct the part played by platelet massing in the formation of the individual mixed thrombus from only one observation. As shown by *Baumgartner, Studer & Reber* (1963) profound structural changes may occur during the establishment of a mixed thrombus, perhaps resulting in a less conspicuous permanent platelet part than what should be expected from the size of the original platelet thrombus.

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The increase of fibrin was succeeded by fibrinolysis both in a general and local form The morphological criteria of general fibrinolysis

have been studied by *Sandritter & Bergerhof* (1954 a and b) and *Sandritter, Bergerhof & Kroker* (1954) The lysis entailed splitting and disappearance of fibrin bands, and the upstream end of the thrombus was first attacked The platelet part of mixed thrombi was more resistant to lysis than the coagulation part These findings are confirmed in the present study It should be added that fragmentation of the peripheral membrane and disappearance of the delicate net allowed a marked escape of erythrocytes, which seemed to be the main cause of the reduction and fissuration of older thrombi When platelet aggregates hidden by fibrin were attacked, the platelets were unmasked, apparently unaffected by the lysis *Wolf* (1961) showed *in vitro* that platelet masses were microscopically unaltered by plasmin digestion, but their mechanical strength was poor

The fibrinolysis did not usually become manifest before the 5th–7th day, earlier and more marked in the thromboplastin-made—and venous—thrombi, than in the toxin-produced—and arterial—thrombi The relative resistance of the toxin-produced thrombi agrees with *Freiman Bang & Cliffton* (1960) who found that thrombi formed by chemical injury to the vascular wall were less amenable to fibrinolytic therapy than coagulation thrombi If staphylokinase was present in the toxin preparation, it does not appear to have played any rôle The greater fibrinolysis in veins may be related to the larger fibrinolytic potency of the venous wall (*Todd* 1958) However, it may also be accounted for by the rich venous collateral circulation through tributaries, lacking in arteries (*Cliffton* 1963) Actually, the fact that the dissolution started at the upstream end of he thrombi, is more in favour of an effect of factors carried with the blood than by local tissue activators

Heparin treatment both hastened and increased the fibrinolysis, in accordance with *Rabinovitch & Pines* (1943), *Loewe, Hirsch & Grayzel* (1947), *Sandritter & Bergerhof* (1954 a), and *Sandritter, Bergerhof & Kroker* (1954) *Wright, Kubik & Hayden* (1952) got the same result by treatment with a prothrombin depressant and ascribed this to a relative preponderance of the fibrinolysis when clotting is impaired Considering the slight effect of heparin on the increase of fibrin, this cannot account for the present observations More likely is a direct stimulation of fibrinolysis by heparin (*Halse* 1947, *von Kaulla & McDonald* 1958) The reduced number of toxin-produced thrombi under heparin treatment may be due to the preventive effect of heparin on the thrombus still not established at the initiation of the therapy The number of the more readily formed thromboplastin-made thrombi was not reduced Thus, the increased fibrinolysis did not definitely result in complete disappearance of the fully developed thrombi However, this may be related to the presence of ligatures which prevented embolization of partly lysed material

Many of the freely floating platelet aggregates observed in vessels with restored circulation may be completely detached thrombi origin-

ally formed on the vascular lining for platelets may adhere and clump even on apparently normal endothelium (*Samuels & Webster* 1952 *Geissinger Mustard & Rowsell* 1962) The mentioned aggregates went through the usual development from a loose collection to a dense granular mass However the most advanced of them had an appearance which did not fit in with an originally adherent platelet thrombus They had a granular centre surrounded by a faint fibrin membrane and only on the outside of this ballooned platelets occurred Aggregates identical to these were produced *in vitro* by tissue thromboplastin and recalcification plus ADP Further, they had great resemblance to the *in vitro* thrombi formed in the moving column of blood in the circulating loop described by *Chandler* (1958) (*Poole* 1959 *French & Poole* 1963 *Chandler* 1964) This favours the concept that the freely floating aggregates in the rabbit vessels were formed in moving blood independent of the wall and point to the importance of an

They may be explained if we assume the formation of a dense aggregate of platelets *Jørgensen & Borchgrevink* (1964) proposed that the ballooning is produced by thrombin on the platelet surface at the height of the thrombin effect When clotting is initiated dense platelet aggregates is conceivably formed as soon as trace amounts of thrombin appears while the clotting on the surface does not become sufficiently stimulated for the creation of ballooning Secondarily this takes place only on the surface of the peripheral platelets In platelet plugs and thrombi the coagulation on the platelet surface is perhaps more strongly triggered as the platelets are caught one by one but even in the thrombi the ballooning was more marked on the outside of the fibrin membrane than on the inside

The particular trend for the freely floating aggregates to form under heparin treatment probably reflects the platelet clumping effect of this substance (*Copley* 1948 *Ashwin* 1961 *Mustard, Murphy, Downie & Rowsell* 1963 *Nordoy & Odegaard* 1963)

SUMMARY AND CONCLUSIONS

Arterial and venous thrombi were produced in rabbits by two methods injection of staphylococcal toxin into the vascular wall combined with partial ligation and injection of tissue thromboplastin into a temporarily isolated section of the vessel One fourth of the animals received heparin treatment after the initiation of the thrombosis The rabbits were sacrificed at varying intervals from ½ hour to 50 days after the injection and the histological changes of the thrombi were followed until they became organized The observations were compared with rabbit platelet plugs and platelet aggregates formed in citrated platelet rich plasma by ADP connective tissue, tissue thromboplastin recalcification and thrombin

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Staphylococcal toxin gave platelet or mixed thrombi, tissue thromboplastin coagulation thrombi The platelet thrombi were located to sites of eddying and underwent the same changes as platelet plugs, usually ending in complete fibrinous transformation of the aggregates Mural thrombi tended to break up and embolize over several weeks Complete occlusion was effected by formation of a coagulation thrombus The coagulation thrombi contained a framework of fibrin membranes running in streamline pattern Platelets were connected with the coagulation thrombi as knots in the fibrin net, as larger aggregates adhering to the membranes, or as secondarily formed platelet thrombi on the free surface There was a steady increase of fibrin, particularly in association with any aggregate of platelets Fibrinolysis started at the upstream end of the thrombi after 5–7 days in non-heparinized animals, after 2–3 days in the heparinized ones The fibrinolysis was more marked in the latter group, while the heparin treatment had only slight influence on the increase of fibrin

The morphological development of platelet plugs in rabbits was slower than in man Aggregates formed by ADP were small and loose, and the platelets granular Connective tissue gave larger, unevenly dense aggregates Aggregates induced by coagulation were dense and granular, tissue thromboplastin and recalcification gave a fringe of ballooned platelets The same picture was found in freely floating platelet aggregates in vessels with restored circulation, particularly in the heparinized animals

The following *conclusions* are drawn

The observations in the earliest stage of platelet thrombosis are compatible with the concept that in the present experiments ADP, and not connective tissue, brings about the initial adhesion and aggregation

The localization and modelling of the platelet thrombi are dependent on haemodynamic factors, particularly the formation of eddies, while retardation of the flow is of minor importance when the local stimulus to platelet thrombosis is strong and enduring

Mural platelet thrombi tend to break up and embolize over an extended period of time

Coagulation thrombi are formed in retarded, but still flowing blood, and their membranes are probably formed at the contact surface between two laminae of flow

Both the increase of fibrin and the fibrinolysis are probably related to plasma infiltration into the thrombus In the former case the local activation of the clotting is probably associated with the platelets, in the latter case local tissue activators probably play a minor role compared with factors carried with the blood

The increased fibrinolysis under heparin treatment is probably caused by direct stimulation of fibrinolysis and is not secondary to impaired clotting

The freely floating platelet aggregates are probably formed by clotting in flowing blood independent of the wall, as in Chandler's loop

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ACTION OF PARAINFLUENZA VIRUS TYPE 3 ON SYNTHESIS OF INTERFERON AND MULTIPLICATION OF HETEROLOGOUS VIRUSES

By

SVANTE HERMODSSON

Received 01 ii 64

Mixed viral infections often lead to one of the viruses inhibiting the multiplication of the other This type of viral interaction has been associated with inhibitors of virus multiplication, interferon substances which are produced by cells exposed to virus (for references see 19) foreign nucleic acids (27) or nucleic acid derivates (21)

It has also been shown that a virus can stimulate the multiplication of another virus (16, 20, 23) The stimulating effect of parainfluenza virus type 3 (PIV-3) on the multiplication of Newcastle disease virus (NDV) seems to be due to PIV-3 inhibiting the production and antiviral action of interferon (16) The effect of PIV 3 on production of interferon and multiplication of heterologous viruses has been further analysed in the present study

MATERIAL AND METHODS

Cell cultures Primary cultures of pig and calf cells were prepared from trypsinized kidney tissue The cells were grown at 37° C in Hanks salt solution supplemented with 0 5 per cent lactalbumin hydrolysate (Hanks + LAH) 10 per cent calf serum and antibiotics (100 I U penicillin and 100 µg streptomycin/ml) The same medium without serum or Eagle s minimal essential medium (MEM) (11) were used for maintenance Cultures of HeLa cells were prepared as described previously (17)

Viruses The PIV 3 strains used had been isolated from cattle or human being The bovine strains were strain 23 (10) strain SF 4 (26) strain V 325 62 (2) strain R 2A (3) strains R 2V9 Sd 2 and FC isolated by Dr *K Bogel* strain T 1 (8) and strain Brookes isolated by Dr *P S Dawson* All these strains had been isolated and transferred in several passages in calf kidney cultures The human strains were strain FA102 (6) and strain 2225 The latter strain was supplied by Dr *F R Abinanti* and had been isolated in monkey kidney cells and was then transferred in 3 passages in calf cells in this laboratory Strain FA102 had been isolated in KB cells and had undergone 5 passages in calf kidney cultures Seed virus suspensions of the PIV-3 strains consisted of culture fluid harvested 3 days after infection of calf kidney cultures with virus at an input multiplicity (im) of about 0 01

The author is indebted to Dr *F R Abinanti* Bethesda Dr *F Burki* Bern Dr *K Bogel* Tubingen Dr *C Chany* Paris and Dr *P S Dawson* Weybridge who kindly supplied different PIV-3 strains A gift of actinomycin D from Dr *L Philipson* Uppsala is gratefully acknowledged

Challenge infections in interference experiments or interferon assays were carried out with strain Sendai of parainfluenza virus type 1, strain Z of pseudorabies virus, strain 80 61 of bovine enterovirus, strain 15 of swine influenza virus and strain F206 of poliovirus type 1 Stock suspensions of Sendai virus, bovine enterovirus and swine influenza virus were prepared in calf kidney cultures and pseudorabies virus was prepared in pig kidney cultures Infectious RNA from poliovirus strain F206 was prepared as described previously (18) The PIV-3 strains were stored at 4° C and the other viruses at —60° C

Virus titrations Infectivity was measured as described earlier (17) by plaque assay in HeLa cells or by endpoint titration in roller tube cultures of calf kidney cells In some experiments infectivity of PIV 3 was assayed in tube cultures inoculated with 0 2 ml of fourfold dilutions of the samples into groups of 10 cultures The titre was then computed by the most probable number method (5)

In mixed viral infections, PIV 3 was neutralized by addition of PIV-3 antiserum as described previously (16)

Assay of haemagglutinin (HA) was carried out as described previously (17)

Assay of interferon Interferon activity was assayed by two different methods Viral activity was removed by dialysis at pH 2 0 ultracentrifugation and addition of antiviral serum as described previously (17) Twofold dilutions of the samples were inoculated into groups of 2 roller tube cultures The cultures were incubated at 37° C for 18 to 24 hours and were subsequently challenged with 10^4 TCD_{50} of Sendai virus After incubation for another 3 to 4 days the media were assayed for haemagglutinin and the titre expressed as the reciprocal of the highest dilution which reduced the HA yield by 75 per cent

When a more sensitive method [illegible] maintenance medium were inocu[illegible] grown in plastic petri dishes Afte[illegible] washed in phosphate buffered sal[illegible] forming units (PFU) of polioviru[illegible] room temperature the cultures we[illegible] added After incubation for 24 [illegible] —60° C The media were subsequently assayed for poliovirus infectivity by plaque titration The interferon titre was calculated on the basis of the observed linear relationship between log reduction of poliovirus yield and log concentration of interferon (18) Since it has not been ascertained whether different interferon preparations give the same regression coefficient the interferon titre was calculated from dilutions which reduced the poliovirus yield 50 to 95 per cent and from a regression of — 0 81 the mean for 9 different titrations The interferon titre was expressed in units representing the reciprocal of the dilution of the sample which reduced the poliovirus yield by 50 per cent

Ultraviolet (UV) irradiation The virus suspension was centrifuged at 15 000 *g* for 60 minutes and the pellet resuspended in 0 15 M NaCl buffered with 0 0[illegible] M [illegible]

RESULTS

Production of Interferon in Calf Cells Infected with Different PIV 3 Strains

In a previous investigation (16) no interferon could be demonstrated in calf kidney cultures infected with strain 23 of PIV-3 It was possible, however, that such cultures produced small amounts of interferon which might have escaped detection The production of interferon was therefore studied by a more sensitive assay method (18) than that used previously

Flask cultures of calf cells were infected with strain 23 (im ~ 0 1), incubated at 37° C and assayed for interferon activity by challenge with

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Mixed viral infections often lead to one of the viruses inhibiting the multiplication of the other. This type of viral interaction has been associated with inhibitors of virus multiplication, interferon substances which are produced by cells exposed to virus (for references see 19), foreign nucleic acids (27) or nucleic acid derivates (21).

It has also been shown that a virus can stimulate the multiplication of another virus (16, 20, 23). The stimulating effect of parainfluenza virus type 3 (PIV-3) on the multiplication of Newcastle disease virus (NDV) seems to be due to PIV-3 inhibiting the production and antiviral action of interferon (16). The effect of PIV-3 on production of interferon and multiplication of heterologous viruses has been further analysed in the present study.

MATERIAL AND METHODS

Cell cultures. Primary cultures of pig and calf cells were prepared from trypsinized kidney tissue. The cells were grown at 37° C in Hank's salt solution supplemented with 0.5 per cent lactalbumin hydrolysate (Hank's + LAH), 10 per cent calf serum and antibiotics (100 I.U. penicillin and 100 µg streptomycin/ml). The same medium without serum or Eagle's minimal essential medium (MEM) (11) were used for maintenance. Cultures of HeLa cells were prepared as described previously (17).

Viruses. The PIV-3 strains used had been isolated from cattle or human beings. The bovine strains were strain 23 (10), strain SF 4 (26), strain Y 325/62 (2), strain R 2A (3), strains R 2V9, Sd 2 and I 6 isolated by Dr. *K. Bogel*, strain T 1 (8) and strain Brookes isolated by Dr. *P. S. Dawson*. All these strains had been isolated and transferred in several passages in calf kidney cultures. The "human" strains were strain FA102 (6) and strain 2225. The latter strain was supplied by Dr. *F. R. Abinanti* and had been isolated in monkey kidney cells and was then transferred in 3 passages in calf cells in this laboratory. Strain FA102 had been isolated in KB cells and had undergone 5 passages in calf kidney cultures. Seed virus suspensions of the PIV-3 strains consisted of culture fluid harvested 3 days after infection of calf kidney cultures with virus at an input multiplicity (im) of about 0.01.

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Challenge infections in interference experiments or interferon assays were carried out with strain Sendai of parainfluenza virus type 1 strain Z of pseudorabies virus strain 80 61 of bovine enterovirus strain 15 of swine influenza virus and strain E206 of poliovirus type 1 Stock suspensions of Sendai virus bovine enterovirus and swine influenza virus were prepared in calf kidney cultures and pseudorabies virus was prepared in pig kidney cultures Infectious RNA from poliovirus strain E206 was prepared as described previously (18) The PIV–3 strains were stored at 4° C and the other viruses at —60° C

Virus titrations Infectivity was measured as described earlier (17) by plaque assay in HeLa cells or by endpoint titration in roller tube cultures of calf kidney cells In some experiments infectivity of PIV 3 was assayed in tube cultures inoculated with 0 2 ml of fourfold dilutions of the samples into groups of 10 cultures The titre was then computed by the most probable number method (5)

In mixed viral infections PIV 3 was neutralized by addition of PIV 3 antiserum as described previously (16)

Assay of haemagglutinin (HA) was carried out as described previously (17)

Assay of interferon Interferon activity was assayed by two different methods

After incubation for another 3 to 4 days the media were assayed for haemagglutinin and the titre expressed as the reciprocal of the highest dilution which reduced the HA yield by 75 per cent

When a mo maintenance grown in pla washed in ph forming unit room temper added After —60° C The media were subsequently assayed for poliovirus infectivity by plaque titration The interferon titre was calculated on the basis of the observed linear relationship between log reduction of poliovirus yield and log concentration of interferon (18) Since it has not been ascertained whether different interferon preparations give the same regression coefficient the interferon titre was calculated from dilutions which reduced the poliovirus yield 50 to 95 per cent and from a regression of —0 81 the mean for 9 different titrations The interferon titre was expressed in units representing the reciprocal of the dilution of the sample which reduced the poliovirus yield by 50 per cent

Ultraviolet (UV) irradiation The virus suspension was centrifuged at 15 000 g for 60 minutes and the pellet resuspended in 0 15 M NaCl buffered with 0 02 M tr(hydroxyme h

RESULTS

Production of Interferon in Calf Cells Infected with Different PIV 3 Strains

In a previous investigation (16) no interferon could be demonstrated in calf kidney cultures infected with strain 23 of PIV 3 It was possible however that such cultures produced small amounts of interferon which might have escaped detection The production of interferon was therefore studied by a more sensitive assay method (18) than that used previously

Flask cultures of calf cells were infected with strain 23 (im ~ 0 1), incubated at 37° C and assayed for interferon activity by challenge with

poliovirus RNA No interferon activity was demonstrated in the medium, when tested in a dilution of 1 4 as shown in Table 1

Other strains of PIV–3 were examined similarly for their ability to *induce production of interferon in calf cells* The results of the interferon assays are given in Table 2 High titres of interferon were found in cultures infected with the strains 2225 and EA102 No or only slight interferon activity was demonstrated in cultures infected with strains SF 4, V 325/62, Brookes, E6, R-2A, R-2V9 and Sd-2, although more haemagglutinin was produced in these cultures than in cultures infected with the strains 2225 and EA102 An interferon activity of 200 units was demonstrated in the medium of cultures infected with strain T 1

TABLE 1

Absence of Interferon Activity in Medium of Calf Kidney Cultures Infected with Strain 23 of PIV 3

Period of incubation in days	Yield of poliovirus in per cent of control — Dilution of culture medium tested		Estimated titre on interferon
	1 4	1 16	
2	100	140	<4
4	126	172	<4
6	67	100	<4

TABLE 2

Yield of Interferon in Calf Kidney Cultures Infected with Different Strains of PIV–3

PIV 3 strain	Yield of poliovirus in per cent of control — Dilution tested									Estimated titre of interferon
	1 4	1 16	1 64	1 256	1 500	1 1024	1 2000	1 8000	1 32000	
SF 4	77	100	110	144		125	–		–	<4
V 325/62	42	96	124	88	–	110		–	–	5
T 1		6 0	42	84		58	–		–	200
Brookes	*81*		*118*	*160*	–	*120*				<4
E 6	87	100	78	–		91		–	–	<4
R 2A	104	100	115			109	–			<4
R 2V 9	41	84	123		–	111				5
Sd 2	83	96	110	–		125				<4
LA102					3 8		16	31	88	10 000
2225	–	0 56	2 9	13		39				1 000

– = *not done*

Influence of Environmental Conditions on Production of Interferon

The influence of temperature on interferon production in PIV 3 infected cultures was studied since *Ruiz-Gomez & Isaacs* (28) have shown an increased yield of interferon at temperatures above the optimal temperature for virus growth

Calf kidney cultures were infected with strain 23 (im ~ 10), and in-

cubated at different temperatures. The medium was assayed for haemagglutinin daily and for interferon after incubation for 4 days. Interferon was assayed by challenge with poliovirus RNA. The production of haemagglutinin was not markedly influenced by variations of the temperature between 33° and 38.5° C, but was inhibited significantly by incubation at 40° C. Interferon activity, however, was not demonstrated in cultures incubated at 40° C or in cultures incubated at the lower temperatures (Table 3).

TABLE 3

Influence of Different Conditions on Production of Interferon in Cultures Inoculated with Strain 23 of PIV 3

Cultures assayed for interferon	Yield of poliovirus in per cent of control						Estimated titre of interferon
	Dilution tested						
	1:4	1:16	1:64	1:256	1:1024	1:4096	
PIV 3 infected calf cultures							
incubated at 33°	72	96	179	103	–		<4
incubated at 37°	67	119	99	96	–		<4
incubated at 38.5°	63	72			–		<4
incubated at 40°	90	73	140	86	–	–	<4
with acid medium pH 6.8	55	96				–	<4
PIV-3 infected pig cultures		4.5	18	39	142	–	300
Calf cultures inoculated with							
heat inactivated PIV 3	2.6	6.9	10	58		–	300
UV irradiated PIV-3	–		0.82	4.6	7.6	53	10 000

– = not done

Slightly acid conditions are known to increase the yield of interferon in rat cells infected with Sindbis virus (9). A similar experiment was therefore carried out with calf kidney cultures infected with strain 23 (im ~ 10). The pH of culture fluids were adjusted to either 6.8 or 7.4 by addition of CO_2. No interferon, however, was demonstrated in infected cultures incubated with acid medium for 3 days (Table 3).

Whether or not infection with strain 23 could induce interferon synthesis in cells other than calf cells was also studied. Pig cells were chosen since these cells produce interferon which can be assayed in calf cells (24). Flask cultures of pig cells were infected with strain 23 (im ~ 10) and incubated for 3 days at 37° C. The medium of these cultures and uninfected controls were subsequently assayed for interferon. No interferon was found in uninfected cultures when the medium was tested in a dilution of 1:16 by challenge with poliovirus RNA. The infected cultures, on the other hand, had an interferon activity of 300 units (Table 3).

Interferon Production by Inactivated Virus

Samples of the stock suspension of strain 23 were incubated at 37° C for 48 hours or irradiated with UV-light for 2 minutes. These treatments reduced the infectivity 3 and 4 log units respectively. The virus sus-

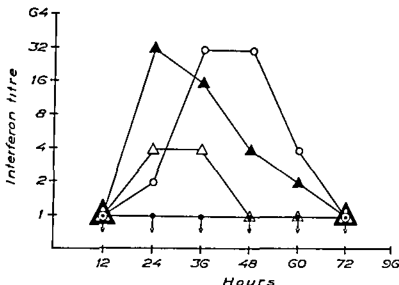


Fig 1.

Production of interferon in calf kidney cultures inoculated with UV irradiated and untreated virus of two different PIV 3 strains

▲——▲ Untreated virus EA102
●——● Untreated virus 23
○——○ UV-irradiated virus 23
△——△ UV-irradiated virus EA102

pensions were inoculated in 2 ml amounts into calf kidney cultures in plastic petri dishes. The cultures were incubated at 37° C for 1 hour, washed and maintenance medium added The culture fluids were then assayed for interferon by challenge with poliovirus RNA after 3 days at 37° C Interferon activity was found both in cultures inoculated with heat-inactivated virus and in cultures inoculated with UV-irradiated virus (Table 3) The highest concentration of interferon was found in cultures exposed to UV-irradiated virus The interferon titre in these cultures was of the same magnitude as in cultures infected with strain EA102 (cf Table 2)

The rate at which interferon was produced in calf cells treated with the strains 23 and EA102 was studied by exposing stock suspensions to UV light for 2 minutes as described in Material and Methods Untreated and irradiated virus was then inoculated into separate plate cultures of calf cells The cultures were washed twice after 1 hour at 37° C and fresh medium added The medium was changed every 12 hours and assayed for interferon activity by challenge with Sendai virus

The results in Fig 1 show that interferon was produced more rapidly in cultures inoculated with untreated virus of strain EA102 than in cultures inoculated with UV-irradiated virus of strain 23 Maximum yields of interferon were obtained after incubation for 24 hours with strain EA102 and for 36 hours with strain 23 Less interferon was obtained with the strain EA102 after UV-irradiation of virus

The influence of UV light on virus 23 was further analyzed by irradiation for various periods After UV-irradiation the virus suspensions

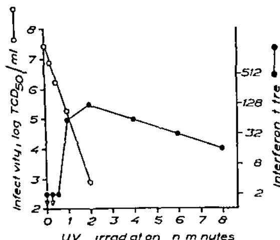


Fig 2
The effect of UV irradiation on infectivity of strain 23 and on interferon production in calf kidney cultures inoculated with this virus

were diluted tenfold in maintenance medium assayed for infectivity and inoculated in 2 ml amounts into plate cultures of calf cells The cultures were washed twice after 1 hour at 37° C and supplied with fresh medium The culture fluids were subsequently assayed for interferon by challenge with Sendai virus after further incubation of the cultures for 2 days at 37° C

The decrease of the infectivity per minute of irradiation was found to be 2 2 log units (Fig 2) Maximum interferon titre was obtained with virus irradiated for 2 minutes The yield of interferon decreased gradually after further irradiation and was 1/8 the maximum yield after irradiation for 8 minutes

The possibility was examined whether the interferon production obtained with UV irradiated strain 23 might be due to infection with an interferon inducing virus e g a UV resistant mutant Strain 23 was UV irradiated for 2 minutes and inoculated into a culture of calf cells The medium of this culture was harvested after 2 days at 37° C and inoculated in serial tenfold dilutions into roller tube cultures of calf cells These cultures were incubated for 5 days at 37° C after which the cells were examined for cytopathic changes and the media for interferon by challenge with Sendai virus The infectivity of the medium from the culture inoculated with UV irradiated virus was found to be $10^{1.5}$ TCD_{50} per ml No interferon was demonstrated in cultures inoculated with this medium in dilutions 10^{-1} to 10^{-4} thus indicating that the interferon production obtained with UV irradiated virus 23 was not induced by infectious virus

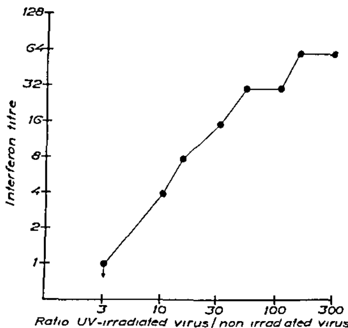


Fig 3
The inhibitory effect of infectious virus on interferon production by UV irradiated virus of strain 23

Effect of Non-Irradiated Virus on Interferon Production by UV-Irradiated Virus

The influence of strain 23 on interferon production was investigated in an experiment in which different dilutions of untreated virus were added to equal volumes of virus UV-irradiated for 2 minutes Calf kidney cultures were inoculated with these suspensions and assayed for interferon after incubation for 2 days at 37° C The yield of interferon was found to decrease at increasing concentrations of non-irradiated virus (Fig 3) No reduction of the interferon yield was demonstrated when the UV-irradiated virus contained less than 1 per cent untreated virus With 10 per cent untreated virus the interferon yield was reduced 16 times and with 30 per cent untreated virus more than 64 times

Inactivation of Interferon in PIV 3 Infected Cultures

It was shown in a previous communication (16) that an infection with strain 23 of PIV–3 inhibits production and antiviral action of interferon in calf cells This effect might be caused by an inactivation of interferon in PIV 3 infected cultures

To study this hypothesis flask cultures of calf kidney cells were infected with strain 23 (im ~ 10) After incubation for 3 days at 37° C the medium was concentrated about tenfold by dialysis against powder of polyethylene glycol 20 M The cells were removed from the glass, sedimented by centrifugation and resuspended in 0 15 M NaCl buffered to pH 7 3 with 0 02 M Tris-buffer This cell suspension which contained

about 5×10^7 cells per ml was disintegrated in a Potter Elvejhem grinder The concentrated medium and the disintegrated cells in volumes of 1.5 ml were then mixed with 0.5 ml NDV interferon (17) or 0.5 ml Hanks + LAH The suspensions were incubated at 37° C for 24 hours and assayed for interferon by challenge with Sendai virus To test if intact cells could destroy interferon cells from infected cultures were overlaid with 5 ml interferon or 5 ml Hanks + LAH incubated at 37° C for 24 hours and assayed for residual interferon activity in the fluid phase Materials from uninfected cultures were treated in the same way

No significant reduction of the interferon titre was obtained when interferon was incubated with intact cells disintegrated cells or concentrated medium from PIV-3 infected cultures (Table 4) This result does not support the hypothesis that interferon is inactivated in calf kidney cultures infected with strain 23 of PIV-3

TABLE 4

Failure to Demonstrate Inactivation of Interferon by Incubation of NDV Interferon with Medium Disintegrated and Intact Cells from Calf Kidney Cultures Infected with Strain 23 of PIV 3

Incubated with	Titre of interferon			
	PIV 3 infected cultures		Uninfected cultures	
	Interferon	No interferon	Interferon	No interferon
Medium	8	<2	4	<2
Disintegrated cells	8	<2	8	<2
Intact cells	8	<2	8	<2

Presence of Incomplete Virus in PIV 3 Infected Cultures

Synthesis of interferon in L cells infected with NDV appears to be induced by incomplete virus (15) If this is also valid for calf cells infected with PIV 3 it could be expected that smaller amounts of incomplete virus are produced in cells infected with strain 23 than in cells infected with strain LA102 since an infection with the latter virus gave rise to more interferon

Calf kidney cultures were infected with these PIV 3 strains (m ~ 0 1) and incubated at 37° C The medium was changed every 12 hours and assayed for infectivity and haemagglutinin Strain LA102 was assayed for infectivity in HeLa cells and strain 23 in roller tube cultures of calf cells The infectivity was expressed in plaque forming units (PFU) for strain LA102 and in most probable number of cytopathic units (MPNCU) for strain 23 The HA titrations were performed both at 4° C and at 37° C Table 5 shows that the ratio between the number of infectious and haemagglutinating units was not significantly higher in cultures infected with strain 23 than in cultures infected with strain LA102 The mean of this ratio was approximately 8×10 when haem

agglutinin was assayed at 4° C and approximately 0 3 × 10^6 when the assay was performed at 37° C Thus noninfectious, haemagglutinating virus does not appear to be produced in high concentration in calf kidney cultures infected with strain EA102

TABLE 5

Yield of Haemagglutinin and Infectious Virus in Cultures Infected with PIV–3 Strains EA 102 and 23

Incubation in hours	HA units ml		PFU ml × 10^{-6}	MPNCL ml × 10^{6}	Ratio infectivity HA × 10^{4}	
	Strain EA 102	Strain 23	Strain EA 102	Strain 23	Strain EA 102	Strain 23
12	<4 (<4)	<4 (<4)	–	–	– –	– –
24	<4 (4)	4 (64)	1 5	23	– (0 38)	5 8 (0 36)
36	8 (128)	16 (512)	63	235	7 9 (0 49)	15 (0 46)
48	4 (128)	32 (512)	37	90	9 3 (0 29)	2 8 (0 18)
60	4 (64)	32 (1024)	13	191	3 3 (0 20)	6 0 (0 19)
72	<4 (32)	32 (1024)	11	441	– (0 34)	14 (0 43)

Figures in brackets refer to HA titres obtained at 37°
– = not done

Interference between PIV–3 and Some Heterologous Viruses

Interference between PIV–3 and heterologous viruses was studied in calf kidney cultures infected with either the strain EA102 or the strain 23 (im ~ 10) The PIV–3 infected cultures and uninfected controls were incubated at 37° C for 1 day and superinfected with 10^4 to 10^5 TCD_{50} of pseudorabies virus, bovine enterovirus, swine influenza virus or Sendai virus After 2 hours at 37° C the infected cultures were washed twice and supplied with fresh medium Samples of the culture fluids were withdrawn at intervals and assayed for infectivity after neutralization of PIV 3 by antiserum

Fig 4 shows that a previous infection with strain EA102 strongly inhibited the multiplication of all viruses tested, whereas strain 23 only inhibited Sendai virus Strain 23 appeared on the other hand to promote the multiplication of pseudorabies virus Evidence for a stimulation of the multiplication of pseudorabies virus was also obtained when the cultures were assayed for pseudorabies infectivity after freezing and thawing

The Action of Strain 23 on the Multiplication of Poliovirus in Calf Cells

To ensure that the observed interference between strain 23 of PIV–3 and parainfluenza virus type 1 was not due to presence of interferon in the cells, the PIV–3 infected cultures were challenged with poliovirus RNA Such an infection has previously been found to be extremely sensitive to interferon (18) Calf kidney cells grown in plastic petri dishes were infected with strain 23 (im ~ 10) and incubated for 1 day

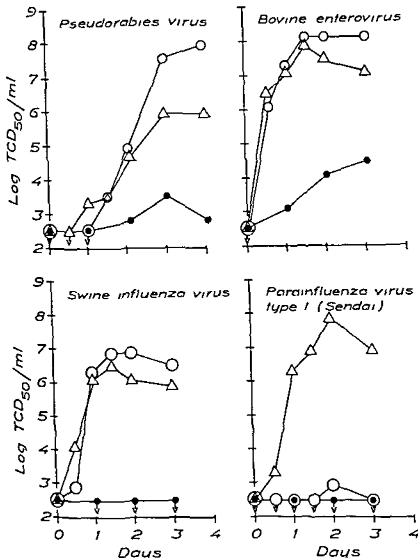


Fig 4

The multiplication of some challenge viruses in calf kidney cultures previously infected with strain 23 (○——○) or strain FA102 of PIV 3 (●——●) Control cultures infected with challenge virus only (△——△)

at 37° C The PIV-3 infected cultures and uninfected controls were then inoculated with 80 PFU of poliovirus RNA according to the technique described in Material and Methods The cultures were incubated at 37° C for 36 hours and assayed for poliovirus infectivity No suppression of the poliovirus occurred in cultures previously infected with PIV 3 (Table 6) This mixed infection yielded 6 times higher titres of

poliovirus in the medium than the single infection with poliovirus RNA. Similar results were obtained when the cultures were assayed after *freezing and thawing*. Nor was it possible to detect higher concentrations of poliovirus in the cell phase than in the medium after treatment of the cells with 8 M urea for 30 seconds at room temperature.

TABLE 6

Yield of Poliovirus in PIV-3 Infected Cultures after Infection with 80 PFU of Poliovirus RNA

Culture	Yield of poliovirus in PFU per culture		
	Medium	Cells + Medium	Urea treated cells
PIV-3 infected	5 600	7 300	1 400
Control	880	1 000	640

The increased yield of poliovirus in cultures infected with strain 23 might be ascribed to the fact that this virus inhibits the production of interferon (16). It was therefore examined whether an increased multiplication of poliovirus also occurred in cultures treated with actinomycin D, since this substance is considered to inhibit the synthesis of interferon (12, 14) but not multiplication of small RNA viruses (25). The effect of actinomycin on interferon synthesis and on a DNA virus was investigated in cultures infected with NDV (im ~ 10) and pseudorabies virus (im ~ 5), respectively. After infection the cultures were incubated for 1 day at 37° C and assayed for infectivity, haemagglutinin and interferon as shown in Table 7.

TABLE 7

The Influence of Strain 23 of PIV 3 and Actinomycin Respectively on the Multiplication of Poliovirus in Calf Kidney Cultures Infected with Poliovirus RNA

Concentration of actinomycin μg/ml	Poliovirus PFU/ml × 10⁻³		Pseudorabies virus Log TCD_{50}/ml		NDV HA units/ml		Interferon Units/5 ml × 10⁻
	PIV 3 infected	Control	PIV 3 infected	Control	PIV 3 infect	Control	NDV infected
0.10	12	0.52	3.5	3.9	64	64	0.8
0.05	9.3	1.6	4.7	5.7	64	64	2
0.025	17	2.8	6.7	7.1			–
0.000	18	3.5	8.1	7.9	32	32	10

The multiplication of poliovirus was found to be inhibited in cultures treated with actinomycin in concentrations exceeding 0.025 μg per ml medium. In the presence of 0.1 μg per ml the poliovirus yield was reduced to 15 per cent of that of controls inoculated with poliovirus RNA only. The production of interferon in NDV infected cultures was also significantly decreased at this concentration of actinomycin although the multiplication of NDV did not appear to be inhibited. An increased

yield of poliovirus was found in actinomycin treated cultures which prior to the inoculation of poliovirus RNA and actinomycin had been infected with strain 23 (Table 7) It was also found that the inhibitory effect of actinomycin on pseudorabies virus was not diminished in cultures previously infected with strain 23

DISCUSSION

In a previous investigation (16) no interferon could be demonstrated in calf kidney cultures infected with strain 23 of PIV 3 Neither was interferon demonstrated in such cultures by a more sensitive method for interferon assay (18) as shown in this study The PIV-3 infected cells were also susceptible to infection with poliovirus RNA which has been found to be sensitive to interferon (18) Furthermore interferon was not demonstrated in PIV 3 infected cultures under conditions which increase the production of interferon in other cell virus system —incubation in slightly acid medium (9) or at temperatures supra optimal for virus growth (28) These results taken together thus indicate that strain 23 can multiply in calf cells without inducing synthesis of interferon

The production of interferon was also studied in calf cells infected with other PIV-3 strains No or only slight interferon activity was obtained with 7 of 8 bovine strains whereas 2 strains of human origin gave rise to high titres of interferon Although no interferon was found in calf cultures infected with the bovine strain 23 interferon was produced by this virus in pig cultures These results support the hypothesis (28) that production of interferon by a virus is not an intrinsic property of the virus particles but a property associated with a particular cell virus interaction The findings also indicate that less interferon is produced by a virus infection when the virus is well adapted to the cells It is of interest in this connection that interferon synthesis is induced by heterologous nucleic acids but not by homologous (27)

An increased production of interferon was obtained in calf kidney cultures inoculated with strain 23 after heat inactivation or UV irradiation of virus Similar results have previously been obtained with other viruses (1 4 13 22) The results obtained with strain 23 indicate that UV irradiation destroys the part of the viral genome responsible for suppression of interferon synthesis Results which supported this hypothesis were (a) interferon production in cultures inoculated with UV irradiated virus did not appear to be induced by infectious virus (b) interferon activity was not destroyed in cultures infected with strain 23 (c) non irradiated virus inhibited production of interferon by UV irradiated virus and (d) an infection with strain 23 inhibited interf

ation of virus is determined by the very loss of

infectivity An evaluation of this question is complicated by the fact that infectious virus even in low concentration inhibited interferon production by UV-irradiated virus, a phenomenon previously described by *Lindenmann* for another cell-virus system (22)

It could not be shown that interferon production in calf kidney cultures infected with the human strain EA102 was due to formation of non-infectious, haemagglutinating virus The ratio infectivity haemagglutinin was approximately 8×10^6 when the HA assay was performed at 4° C The same ratio was obtained for strain 23 Furthermore, less interferon was produced by UV-irradiated virus of strain EA102 than by non-irradiated virus These results therefore indicate that the difference in interferon-inducing capacity between the strains 23 and EA102 might be due to different ability of the viruses to inhibit production of interferon

Although no interferon was found in calf kidney cultures infected with strain 23, such cultures showed a high resistance against infection with Sendai virus No interference was found, on the other hand, between strain 23 and such unrelated viruses as bovine enterovirus, pseudorabies virus, swine influenza virus and poliovirus Since Sendai virus is less sensitive to the antiviral action of interferon in calf cells than poliovirus RNA (18) these results indicate that interference occurs between heterologous but related viruses in the absence of interferon Similar findings has been shown by *Crowell & Syverton* in another cell virus system (7)

Furthermore, an infection with strain 23 stimulated the multiplication of poliovirus in calf cells infected with poliovirus RNA Since the strain 23 can stimulate the multiplication of heterologous virus by inhibiting the production and antiviral action of interferon (16) the enhanced multiplication of poliovirus might be due to this effect However, the yield of poliovirus was decreased in cultures treated with actinomycin, although this compound inhibited production of interferon An infection with strain 23 also enhanced the multiplication of poliovirus in actinomycin-treated cultures It is therefore possible that strain 23 stimulates the multiplication of poliovirus by another mechanism than by inhibition of interferon production

SUMMARY

No or only slight interferon activity was found in calf kidney cultures infected with some bovine strains of parainfluenza virus type 3 (PIV-3), whereas high titres of interferon were obtained by infections with PIV-3 strains of human origin An increased production of interferon was obtained with the bovine strain 23 after UV-irradiation of virus, but not with the human strain LA102 The production of interferon in cultures treated with UV irradiated virus was inhibited by presence of

non irradiated virus of strain 23 This inhibition increased at higher concentrations of untreated virus

Different types of interaction between PIV-3 and heterologous viruses are described Interference mediated by interferon seems to occur between strain EA102 and unrelated viruses as pseudorabies virus and bovine enterovirus, interference without interferon between strain 23 and Sendai virus, and stimulation of virus multiplication in cultures concurrently infected with strain 23 and poliovirus RNA The stimulating effect of strain 23 on the multiplication of poliovirus in calf cells does not appear to be due to an inhibited production of interferon

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infectivity. An evaluation of this question is complicated by the fact that infectious virus even in low concentration inhibited interferon production by UV-irradiated virus, a phenomenon previously described by *Lindenmann* for another cell-virus system (22).

It could not be shown that interferon production in calf kidney cultures infected with the human strain EA102 was due to formation of non-infectious, haemagglutinating virus. The ratio infectivity:haemagglutinin was approximately 8×10^6 when the HA assay was performed at 4° C. The same ratio was obtained for strain 23. Furthermore, less interferon was produced by UV-irradiated virus of strain EA102 than by non-irradiated virus. These results therefore indicate that the difference in interferon-inducing capacity between the strains 23 and EA102 might be due to different ability of the viruses to inhibit production of interferon.

Although no interferon was found in calf kidney cultures infected with strain 23, such cultures showed a high resistance against infection with Sendai virus. No interference was found, on the other hand, between strain 23 and such unrelated viruses as bovine enterovirus, pseudorabies virus, swine influenza virus and poliovirus. Since Sendai virus is less sensitive to the antiviral action of interferon in calf cells than poliovirus RNA (18) these results indicate that interference occurs between heterologous but related viruses in the absence of interferon. Similar findings has been shown by *Crowell & Syverton* in another cell-virus system (7).

Furthermore, an infection with strain 23 stimulated the multiplication of poliovirus in calf cells infected with poliovirus RNA. Since the strain 23 can stimulate the multiplication of heterologous virus by inhibiting the production and antiviral action of interferon (16) the enhanced multiplication of poliovirus might be due to this effect. However, the yield of poliovirus was decreased in cultures treated with actinomycin, although this compound inhibited production of interferon. An infection with strain 23 also enhanced the multiplication of poliovirus in actinomycin treated cultures. It is therefore possible that strain 23 stimulates the multiplication of poliovirus by another mechanism than by inhibition of interferon production.

SUMMARY

No or only slight interferon activity was found in calf kidney cultures infected with some bovine strains of parainfluenza virus type 3 (PIV–3), whereas high titres of interferon were obtained by infections with PIV–3 strains of human origin. An increased production of interferon was obtained with the bovine strain 23 after UV-irradiation of virus, but not with the human strain EA102. The production of interferon in cultures treated with UV-irradiated virus was inhibited by presence of

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STUDIES ON TRANSFORMATION IN MORAXELLA AND ORGANISMS ASSUMED TO BE RELATED TO MORAXELLA

2 *Quantitative Transformation Reactions between* Moraxella nonliquefaciens *Strains, with Streptomycin Resistance Marked DNA*

By

KJELL BØVRE

Received 21 iii 64

The relationships in terms of transformation between strains of the same species, classified according to conventional criteria, are of interest a o for the following reasons

1 For comparative transformation studies it is necessary to have strains which are representative of their species
2 Information concerning the level and general distribution of interstrain transformation rates within a species is necessary for the estimation of a first degree interstrain relationship in terms of transformation An intraspecies transformation rate unit of this kind is fundamental for the evaluation of transformation ratios for eventual classification purposes
3 As a consequence of homogeneity or heterogeneity in terms of transformation the taxonomic significance of minor phenotypic deviations may be better understood

A relatively broad study of the assumed species is preferable for the elucidation of these aspects Previous reports on heterologous transformation reactions in different groups of bacteria partly suffer from the lack of evidence that the strains applied are representative for the species in question It may also be found that the descriptions of the strains used in such studies are insufficient A basis of specified conventional diagnostic criteria is, in the opinion of the author, indispensible for the eventual use of transformation in taxonomy In the absence of adequate information of this kind, the genetic findings will have questionable validity and few practical consequences The use of type culture collection strains without proper control may be misleading (see below)

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28 *Ruiz Gomez J & Isaacs A* Interferon production by different viruses Virology *19* 8 1963

gularly 15–20 µg per ml of the reaction mixture. Controls for DNA saturation consisted of parallels with 30–40 µg DNA per ml which never gave means of transformant scores significantly deviating from those at the lower DNA concentration except for some cases with prolonged DNA exposure.

In attempts to transform the two mentioned recipients with DNAs from the strains 19116/51 and 7552/52 there were also included experiments with continuous DNA exposure (without DNase treatment) during the expression period. In continuous DNA exposure DNA is acting in fluid medium for maximum 30 min. Then aliquots are plated and allowed to reach the stage of pin point colonies before exposure to streptomycin. There is no further DNA addition during incubation of the plates. As even an approximate quantitation under these conditions is most difficult no attempt has yet been made towards the exact fixation of transformation ratios based on continuous DNA exposure. However transformation of *Moraxella nonliquefaciens* 7784 with DNA from a strain of *Moraxella bovis* has shown that continuous DNA exposure gives more than a hundredfold of transformants in relation to the initial number of recipient bacteria as compared with 30 min DNA exposure. This estimation has been made with the usual initial DNA concentration 20 µg per ml.

The strains 19116/51 and 7552/52 were also applied as recipients in transformation experiments using their own mutant DNA and DNA from other strains as transforming principle. In these experiments the same transformation procedure was applied although neither of the two strains had been studied in connection with the methodological analysis (Bøvre 1964).

Transformants were regularly scored at the concentration 500 µg streptomycin

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RESULTS

Description of the Strains

Microscopically the strains revealed plump rods often very short diplobacilli occasionally occurring in long chains. The strain 7552/52 was not as plump as the other strains. There was often a considerable variation in size and shape in the same microscopical preparation. Giant and filamentous forms were not seldom found in the young cultures. This feature was more pronounced in older cultures.

As a rule the bacteria seemed to divide in one plane. In some few of the preparations however there were infrequent occurrences of groups of 4 organisms which might perhaps have arisen by division in two opposite planes. All strains were Gram negative. The general impression was some resistance to decolorization. Irregular staining with strongly stained and pale cells was observed in some preparations. The morphological features of some of the strains are presented in Figs. 1–3.

The colonies on blood agar were of small or moderate size, regularly a little larger than 1 mm in diameter after 20 h incubation. They were round hemispherical or almost flat, often with a domed center and a flat periphery. There was no pigmentation. All variations between

MATERIAL AND METHODS

Moraxella nonliquefaciens has been chosen as the starting material of these studies, because of its frequent occurrence in human material (*Henriksen* 1958 *Kaffk & Blodorn* 1959 a o), and because this species seems to be relatively 'clearcut" from the diagnostic point of view

21 strains of *Moraxella nonliquefaciens* isolated in Oslo from clinical human material were included The bacteriological diagnosis had been made by different bacteriologists during routine examinations, and the diagnosis had been based on the genus description proposed by *Henriksen* (1952) with the addition of the following characters No haemolysis no liquefaction of serum and no serum requirement In addition to these 21 strains, 1 strain was received from The National Collection of Type Cultures, London under the name of *Moraxella lacunata* 7784 It was shown that this strain neither liquefied coagulated bovine serum nor required serum for growth, so that it could be added to the material of *Moraxella nonliquefaciens* strains

The strains had been stored in the lyophilized state at +5° C and were kept in subculture on blood agar for long periods They were examined in the following way, partly as a consequence of transformation results which revealed the need for additional biochemical reactions and cultural characteristics distinguishing between strains with deviating transformation ratios

20 h old blood agar surface cultures, cultivated at 32–33° C in a humid atmosphere were used for microscopical examinations characterization of colony consistency for testing the ability of being emulsified in physiological saline and for detection of 1 per cent aqueous solution of was tested microscopically an h 0 4 per cent agar Surface cultures on Hugh & Leifson s medium were made after the addition of 1 per cent agar to the medium described by *Hugh & Leifson* (1953) The medium was prepared with Bacto-Peptone Acid production from glucose was tested on glucose ascites agar slants (with 1 per cent glucose 24 per cent ascites and phenol red indicator) Utilization of citrate as the sole source of carbon was read in Koser s medium (*Koser* 1924 medium no [2]) Liquefaction of serum was tested on 92 per cent bovine serum in broth coagulated at 80° C for 2 h The test for hydrogen sulphide production was performed in a meat extract peptone agar medium with 0 1 per cent lead acetate The medium for indol production consisted of 2 per cent peptone (Pepton aus Casein tryptisch verdaut Merck) and 0 2 per cent Na_2HPO_4 in distilled water and the medium for nitrite production from nitrate consisted of 0 02 per cent nitrite free KNO_3 and 0 5 per cent Bacto Peptone in distilled water The urease test was performed according to *Blake Christensen* (1946) with a very heavy inoculum from young blood agar cultures

Gelatin liquefaction was not tested because of difficulties in assessing growth in the conventional medium

Readings of growth on Hugh & Leifson s medium and in citrate glucose utilization hydrogen sulphide production indol production and urea decomposition were made after 4 days incubation Readings of nitrite production were performed daily for 4 days and a distinct red colour reaction on at least one occasion with the conventional sulphanilic acid alphanaphtylamine reagent was considered a positive result Remaining nitrate in negative reactions was tested for with the same reagents after reduction by means of Zn powder All incubations were undertaken at 32 33° C except for some growth experiments which were run in parallel at 37° C

Antibiotic sensitivity tests were performed according to the method of *Ericsson Hogman & Wickman* (1954) further described by *Ericsson* (1960) and commented by *Bovre* (1962)

Streptomycin sensitive wild type *Moraxella nonliquefaciens* 4663/62 and 7784 were exposed to DNA from streptomycin resistant mutants of each of the 22 strains all selected at 500 μg streptomycin per ml The methods of mutant selection DNA extraction and quantitative transformation have been described previously (*Bovre* 1964) In the latter communication the above mentioned recipients were both described in detail with regard to their behaviour in the transformation system Regularity of results reflecting methodological stability should therefore be the best obtainable As a rule the DNA exposure was allowed to proceed for 15 min, with DNase treatment at the end of exposure The concentration of DNA was re

gularly 15–20 µg per ml of the reaction mixture Controls for DNA saturation consisted of parallels with 30–40 µg DNA per ml which never gave means of transformant scores significantly deviating from those at the lower DNA concentration except for some cases with prolonged DNA exposure

In attempts to transform the two mentioned recipients with DNAs from the strains 19116 51 and 752/52 there were also included experiments with continuous DNA exposure (without DNase treatment) during the expression period In continuous DNA exposure DNA is acting in fluid medium for maximum 30 min Then aliquots are plated and allowed to reach the stage of pin point colonies before

per ml

The strains 19116 51 and 752 52 were also applied as recipients in transformation experiments using their own mutant DNA and DNA from other strains as transforming principle In these experiments the same transformation procedure was applied although neither of the two strains had been studied in connection with the methodological analysis (Bøvre 1964)

Transformants were regularly scored at the concentration 500 µg streptomycin per ml H ... -mation of strain 7784 with DNA from ... and 10 µg streptomycin per ml were ... eriment intrastrain transformants in ... 00 50 and 10 µg streptomycin per ml The same procedure was applied to the recipient strain 4663/62 with the addition of the selection concentration 1000 µg of streptomycin per ml

Transformants were controlled for resistance to streptomycin by replica plating This method was also applied for the determination of the uniformity of high streptomycin resistance in some of the donor strains

RESULTS

Description of the Strains

Microscopically, the strains revealed plump rods, often very short diplobacilli, occasionally occurring in long chains The strain 752 52 was not as plump as the other strains There was often a considerable variation in size and shape in the same microscopical preparation Giant and filamentous forms were not seldom found in the young cultures This feature was more pronounced in older cultures

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TABLE 1

Some Characteristics and the Origin of the Assumed Moraxella nonliquefaciens Strains

Strain**	Microscopical picture	Consistency of colonies	Agglutinability in physiological saline	Growth on the surface of Hugh & Leifson's medium	Growth in citrate	Nitrite production	Urease activity	Origin
672/58	Usual*	Mucoid	—	—	—	+	—	Sputum, ozaena
2770/60	Usual	Soft	—	—	—	+	—	Nose, ozaena
153/62	Usual	Intermediate§	(+)	—	—	+	—	Nose
19116/51	Usual	Soft		+	+	—‖	—	Clinical material
7784	Usual	Soft	—	—	—	+	—	NCTC, London
4378/62	Usual	Soft	—	—	—	+	—	Nose
3828/60	Usual	Mucoid	—	—	—	+	—	Nose, ozaena
4235/62	Usual	Soft	—	—	—	+	—	Nose
836/61	Usual	Intermediate	(+)	—	—	+	—	Nose
178/62	Usual	Soft	—	—		+	—	Nose
752/52	Smaller diameter†	Mucoid	—	+	—	+	+	Clinical material
270/60	Usual	Mucoid	—	—	—	+	—	Nose
826/61	Usual	Soft	—	—		+	—	Nose
13015/62	Usual	Soft	—	-	—	+	—	Nose
4626/62	Usual	Soft	—	—	—	+	—	Nose
4663/62	Mainly coccoid°	Intermediate	(+)	—	—	+	—	Nose
4863/62	Long chains of cocci	Soft	—	—	—	+	—	Nose
13385/62	Usual	Soft	—	—	—	+	—	Nose
13536/62	Usual	Intermediate	(+)	—	—	+	—	Nose
5030/62	Usual	Friable	+	—	—	+	—	Nose
5058/62	Usual	Friable	+		—	+	—	Nose
1962/62	Usual	Soft	—	—	—	+	—	Clinical material

** Denominator of designation indicates year of isolation
* Cells mainly as presented in Fig. 1 † See Fig. 3 ° See Fig. 2 § Intermediate between soft and friable
‖ Nitrate apparently not attacked as shown by means of Zn powder reduction

opaque and clear colonies could be observed. Most of the strains mainly yielded colonies with a moderately opaque center and an almost clear periphery. Some strains had domed, greyish white colonies, indistinguishable from the common appearance of *Neisseria catarrhalis* colonies. In addition, two of these strains (5050/62 and 5058/62) regularly had friable colonies, which could not be completely emulsified in physiological saline. Other strains had colonies intermediate between friable and soft, and some grew with mucoid colonies. Most strains, however, grew with soft colonies, easily emulsified in saline (Table 1). All strains were nonhaemolytic. The strain 752/52, however, gave a greenish discolorization of the blood agar on prolonged incubation.

The delicacy of growth of these organisms on blood agar was almost similar to the growth characteristics of enterococci on the same medium. The strains 7784 and 752/52 were slightly more slow-growing than the other strains. All strains grew at least as well at 32–33° C as at 37° C on blood agar aerobically, some strains with a slight preference for the lower temperature. In a dry atmosphere at 37° C growth of some strains was retarded as compared with growth in a humid atmosphere at the same temperature. In fluid media 32–33° C also seemed to be a slightly better growth temperature than 37° C. All strains failed to grow anaerobically on blood agar and in fluid media. In 0.4 per cent Brain Heart Infusion agar stab culture all strains grew fairly well down to 5 mm below the surface. All strains grew well on ascites agar and bovine serum. As a rule the strains were not able to grow on Hugh & Leifson's medium (Table 1). Growth in the medium for hydrogen sulphide production was observable with some difficulties in all strains. All strains grew feebly in the indol medium described and somewhat better in the medium for nitrite production.

The strains were all immotile and oxidase positive, and did not produce acid from glucose, liquefy serum, produce indol or hydrogen sulphide. They were all strongly sensitive to penicillin, streptomycin, chloramphenicol, oxytetracycline and erythromycin (Table 2). Other characteristics and the origin of the strains are collected in Table 1.

TABLE 2

Sensitivity to Antibiotics of the 22 Strains Classified as Moraxella nonliquefaciens

Antibiotic	Range of inhibition zones in mm	Approximate range of minimum inhibitory concentrations (m.i.c.)*
Penicillin	33–43	0.07–0.004 I.U./ml
Streptomycin	23–29	1–0.08 µg/ml
Chloramphenicol	30–42	0.5–0.03 µg/ml
Oxytetracycline	24–34	0.6–0.02 µg/ml
Erythromycin	29–40	0.5–0.02 µg/ml

* M.i.c. values calculated from zone diameters by means of regression equations for each antibiotic (*Ericsson* 1960).

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3828/60	Usual	Mucoid	—	—	—	+	—	Nose ozaena
4235/62	Usual	Soft	—	—	—	+	—	Nose
836/61	Usual	Intermediate	(+)	—	—	+	—	Nose
178/62	Usual	Soft	—	—	—	+	—	Nose
752/52	Smaller diameter†	Mucoid	—	+	—	+	+	Clinical material
270/60	Usual	Mucoid	—	—	—	+	—	Nose
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Oxytetracycline	24 34	0 6 0 02 µg/ml
Erythromycin	29 40	0 5 0 02 µg/ml

* M i c values calculated from zone diameters by means of regression equations for each antibiotic (*Ericsson* 1960)

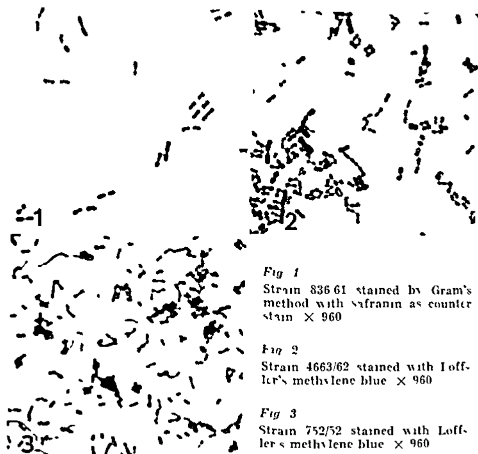


Fig. 1
Strain 836/61 stained by Gram's method with safranin as counter stain. × 960

Fig. 2
Strain 4663/62 stained with Löffler's methylene blue. × 960

Fig. 3
Strain 752/52 stained with Löffler's methylene blue. × 960

Transformation Reactions

The transformation frequency for each donor—recipient combination was compared with simultaneously performed transformation of the recipient with its own mutant DNA, and the ratios of interstrain to intrastrain transformation were determined. The results are collected in Tables 3 and 4. It is evident that 20 out of the 22 strains show a rough uniformity as regards interstrain transformation, the ratios of inter- to intrastrain transformation ranging from $3.4 \cdot 10^{-1}$ to $9.9 \cdot 10^{-1}$. Two strains are definitely deviating in terms of transformation, neither of their DNAs being able to transform members of the intertransformable group. That the DNA of the 19116/51 donor is in its active state, is shown in Table 5. The recipient strain 19116/51 is transformed by its own mutant DNA, but neither by DNAs from the intertransformable group nor from strain 752/52. The strain 752/52 was not transformable, and therefore the activity of its mutant DNA could not be sufficiently controlled. The physical and chemical condition of this DNA did not, however, distinguish itself from other DNAs as regards viscosity and concentration estimated in the Dische diphenylamine reaction.

In a separate experiment (not tabulated) a potent recipient culture

TABLE 3

Ratios of Interstrain to Intrastrain Transformation to Streptomycin Resistance in Presumptive Moraxella nonliquefaciens *Strains*
Recipient Moraxella nonliquefaciens 7784

Donor strain	Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml*	Ratio of inter- to intrastrain transformation
672 58	1 4 10	1 3 10^{5} (127)†	2 4 10^{5} (242)	5 3 10^{-1}
2770 60	6 0 10^{6}	9 4 10^{4} (94)	1 4 10^{5} (143)	6 6 10^{-1}
159 62				not tested
19116 51	1 5 10^{8}	< 10^{1} (0)§	4 7 10^{5} (47)	< 2 1 10^{-5}
4378 62	1 1 10	7 9 10^{4} (79)	2 1 10^{5} (210)	3 8 10^{-1}
3828 60	6 0 10^{6}	9 0 10^{4} (90)	1 4 10^{5} (143)	6 3 10^{-1}
4235/62	1 1 10	1 1 10^{5} (114)	2 1 10^{5} (210)	5 4 10^{-1}
836 61	1 4 10^{7}	1 1 10^{5} (105)	2 4 10^{5} (242)	4 3 10^{-1}
178/62	6 0 10	8 4 10^{4} (84)	1 4 10^{5} (143)	5 9 10^{-1}
752/52	1 5 10^{8}	< 10^{1} (0)§	4 7 10^{5} (47)	< 2 1 10^{-5}
270/60	1 1 10^{7}	9 5 10^{4} (95)	2 1 10^{5} (210)	4 5 10^{-1}
826 61	6 0 10^{6}	1 2 10^{5} (115)	1 4 10^{5} (143)	8 0 10^{-1}
13015/62	1 1 10^{7}	7 7 10^{4} (77)	2 1 10^{5} (210)	3 7 10^{-1}
4626 62	1 1 10^{7}	8 3 10^{4} (83)	2 1 10^{5} (210)	4 0 10^{-1}
4663/62	1 1 10	8 3 10^{4} (83)	2 1 10^{5} (210)	4 0 10^{-1}
4863/62	1 4 10^{7}	1 7 10^{5} (172)	2 4 10^{5} (242)	7 1 10^{-1}
13385/62	2 3 10^{7}	2 0 10^{5} (204)	3 1 10^{5} (307)	6 6 10^{-1}
13536 62	2 3 10^{7}	3 0 10^{5} (303)	3 1 10^{5} (307)	9 9 10^{-1}
5650 62	2 3 10^{7}	1 6 10^{5} (159)	3 1 10 (307)	5 2 10^{-1}
5058 62	2 3 10	2 4 10^{5} (235)	3 1 10^{5} (307)	7 7 10^{-1}
1962 62	6 0 10^{6}	1 1 10^{5} (106)	1 4 10^{5} (143)	7 4 10^{-1}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml
* Intrastrain transformants have been scored in simultaneous transformation of the recipient with its own mutant DNA
† Figures in brackets indicate means of three (in a few cases two) plate counts
§ In parallel continuous DNA exposure transformants were still not detected

of strain 7784 (30 min exposure to homologous DNA giving 3 2 10 transformants per ml at each of the transformant selection concentrations 50 and 10 µg streptomycin per ml) was exposed continuously to DNA from strain 752 52 No interstrain transformants were scored at 50 or 10 µg streptomycin per ml verifying the results from transformation at the 500 µg selection concentration

The other two supplementary experiments which were undertaken in intrastrain transformation of the strains 7784 and 4663 62 to discover eventual heterogeneity or deviation of the degree of streptomycin resistance in transformants similarly did not reveal any differences in the counts of transformants with selection concentrations varying from 1000 to 10 µg streptomycin per ml

On no occasion transformants were found which did not grow freely on the surface of blood agar with 1000 µg streptomycin per ml All donor strains were able to grow at this concentration of streptomycin when simply streaked on the surface Replica plating of 100 colonies of each of the donors 7784 and 4378 62 without exception yielded growth at 1000 µg streptomycin per ml

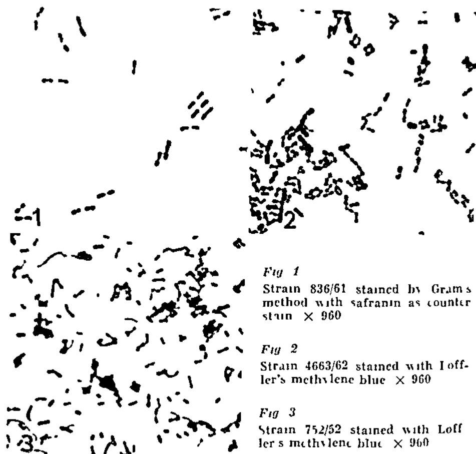


Fig 1
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In a separate experiment (not tabulated) a potent recipient culture

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TRANSFORMATION OF STREPTOMYCIN-RESISTANCE IN BORDETELLA PERTUSSIS[1]

By

P BRANEFORS

Received 25 iii 64

A change in the properties of a bacterial strain can sometimes be obtained by means of exposure of the bacteria to DNA from a related strain

During the last decade this technique, transformation, has become an important tool in microbial genetics *Diplococcus pneumoniae* (8, 9), *Haemophilus influenzae* (2, 3, 10), *Neisseria meningitidis* (6), and a few other species have been studied in this respect Pneumococci and *H influenzae* are probably the best studied species so far A few years ago transformation was also found in conjunction with *Bacillus subtilis* (11, 12, 13), a species that has many well-defined "markers" and consequently promises to be a good system for further genetic investigations

The present paper reports transformation in *Bordetella pertussis*, a species that has not previously been studied from this point of view A streptomycin-sensitive strain of *B pertussis* has been rendered streptomycin resistant by means of exposure to DNA from a resistant variant of the same strain

MATERIAL AND METHODS

Strains *Bordetella pertussis* strain 44122 7 was used as recipient in most experiments It is a virulent (phase I) strain which is used at the State Bacteriological Laboratory Stockholm Sweden for vaccine production This strain is sensitive to streptomycin in concentrations over 1 µg per ml on solid media

The author wishes to express her thanks to Dr *G Heden* for many discussions and for his great interest in the work She also wishes to thank Dr *J Spizizen* for his helpful advice concerning the experimental conditions

[1] The research reported here has been made possible through the support and sponsorship of *the U S Department of the Army* through its European Research Office Contract number DA 91 591 EUC 947 01 1058 59

Moraxella nonliquefaciens These observations are at least promising as regards the use of transformation in classification

SUMMARY

22 bacterial strains, classified as *Moraxella nonliquefaciens,* were investigated, using conventional diagnostic criteria and quantitative streptomycin resistance transformation

20 strains behaved in transformation as closely related strains, with ratios of inter- to intrastrain transformation ranging from 1/3 to 1, approximately Members of this group of strains are considered representative for *Moraxella nonliquefaciens* for the use in transformation studies extended to other bacteria in the taxonomic vicinity of this species

2 strains deviated definitely in terms of transformation These strains both revealed distinct cultural and biochemical characteristics which usually are not recorded or paid special attention to in the diagnosis of *Moraxella nonliquefaciens*

The results are considered promising for the use of transformation as an aid in the classification of these bacteria

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The donor strain was a streptomycin resistant mutant of the same str[illegible]

[illegible] ... discussions and [illegible] ... *Spizizen* for his [illegible]

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EXPERIMENTAL

In the transformation experiments the liquid medium mentioned above was used with the omission of the casamino acids and $CuSO_4$. When performing the experiments a solution of vitamin free casein hydrolysate was added.

Strain 44122/7 was grown from a free dried culture at 35° C for 48 hours on the solid medium mentioned above. It gave a good confluent growth. The same agar slant could be used for about fourteen days if stored at 4° C. When performing an experiment one small loopful of bacteria was transferred to a 100 ml Erlenmeyer flask containing 10 ml of liquid medium to which 1 per cent casein hydrolysate had been added. It was incubated at 37° C on the rotary shaker until it had reached a density of about 1×10^9 cells per ml. This took 18 to 30 hours. From this preculture 1 ml portions were transferred to a number of 100 ml flasks each containing 10 ml of liquid medium to which varying amounts of casein hydrolysate had been added from 0.03 per cent to 1 per cent. These cultures which were intended to develop competence for transformation were incubated on the rotary shaker for

The volume of the test samples usually was 2 ml. To each sample 0.1 ml of DNA was added diluted so that the final DNA concentration was approx. 1 μg per ml sample. The samples in wide test tubes were placed at 37° C on the rotary shaker in order to permit cellular incorporation of DNA varying from 4 to 12 hours. Resistant mutants were screened for by transferring 0.1–0.5 ml of the cultures to the surface of the solid medium containing 200 μg streptomycin per ml. The agar slants were incubated for 4 to 5 days at 35° C.

Diagram Showing the Steps in Transformation

Recipient from lyophilized culture to solid medium (48 hours)

↓

Preculture: 1 loopful to 10 ml of liquid medium containing 1 per cent casein hydr. (18–30 hours)

↓

Competence culture: 1 ml to 10 ml of liquid medium containing 0.03–1 per cent casein hydr. (10–36 hours)

↓

1 ml samples centrifuged at intervals

↓

Sediment diluted to 10 ml with liquid medium containing 0.03–1 per cent casein hydr.

↙ 2 ml treated with DNA
↓ Incorporation of DNA 4–12 hours at 37° C
↓ Solid medium with 200 μg of streptomycin for 4–5 days

↘ 2 ml without addition as control
↓ 4–12 hours at 37° C.
↓ Solid medium with 200 μg of streptomycin for 4–5 days

As mentioned above the negative controls consisted of 2 ml of the remaining centrifuged and diluted culture. Apart from the omission of the DNA these control samples were treated in the same manner as the test samples.

RESULTS

Initially the transformation experiments were performed with the liquid medium that was later used only for DNA preparation. Under these experimental conditions resistant mutants were obtained only rarely.

An increase in the number of transformed bacteria was in some cases

Bacto Casamino acids (Difco technical)	10 g
Yeast extract (Difco)	2 5 g
Soluble starch (Merck)	1 5 g
$MgCl_2$ 6 H_2O 25% solution	1 6 ml
$CaCl_2$ 10% solution	0 1 ml
$FeSO_4$ 7 H_2O 1% solution	1 0 ml
$CuSO_4$ 5 H_2O 1% solution	0 05 ml
l-arginine 0 01% solution	1 0 ml
l histidine 0 005% solution	1 0 ml
KH_2PO_4	0 5 g
	1000 ml aq dest
Ph 7 8	

The agar slants were prepared by adding 1 5 per cent Bacto agar (Difco) to the liquid medium together with 3 per cent of a 7 per cent stock solution of starch After serilization 10 per cent human blood was added

Preparation of DNA (modified from *Spizizen* (12)), strain 44122/7 R was grown on the solid medium for 48 hours at 35° C transferred to 500 ml of liquid medium in a 2 l Erlenmeyer flask, and grown on a rotary shaker (120 rpm stroke excentricity 2 5 cm) at 37° C for 30 hours The whole culture was then poured into a 6 l Erlenmeyer-flask containing 2 5 l of fresh medium and left on the rotary shaker for 48 to 60 hours After this time the culture was in the stationary growth phase and had reached a concentration of at least 1×10^{10} bacteria per ml Two to three such cultures were used for each preparation of DNA The cells were centrifuged in an IEC serumcentrifuge for about 45 minutes (ca $2000 \times g$), and then washed with about 200 ml of 0 1 M NaCl containing 0 015 M Na citrate This washing and the extractions and precipitations reported in the following were carried out at 4° C, except where otherwise stated The bacterial suspension was centrifuged for 20 minutes in an IEC refrigerated centrifuge at $3900 \times g$ The washing and centrifuging of the cells was repeated once The cells were then suspended in the smallest possible amount of 0 1 M NaCl containing 0 015 M Na citrate and transferred to a freeze press (*Edebo* (7)) for disintegrating

e crushed cells were suspended in
l Na citrate and again centrifuged
g for 20 minutes The supernatant
75–100 ml of 2 NaCl in order to
l to facilitate the dispersion The

suspension was recentrifuged at $25000 \times g$ for 20 minutes The upper layer containing most of the DNA was pipetter off and slowly poured into 4 volumes of 95 per cent ethanol The DNA precipitated as a white gelatinous mass which became filamentous when stirred with a glass rod the filaments winding around the rod The material was dried on filter paper Another two similar extractions of DNA with 2 M NaCl were made from the bacterial residue The total quantity of precipitated DNA was then dissolved in about 100 ml of 2 M NaCl and left overnight

The crude DNA preparation was further purified from protein by the addition of 0 1 per cent sodium desoxycholate (2 per cent solution in proportion 1 20) The mixture was allowed to stand for 2 hours at 37° C and 1 hour at 4° C and was then centrifuged at $3900 \times g$ for 20 minutes The supernatant liquid was poured into 4 volumes of sterile 95 per cent ethanol As previously the precipitated DNA was removed and dissolved in a small volume of sterile 2 M NaCl

In some cases this relatively crude preparation of DNA was purified from RNA by digestion with RNAase (Worthington 3 × cryst 50 µg per ml solution of DNA) at 37° C for 1 hour and from protein by the addition of 0 1 per cent sodium desoxycholate at 37° C for 1 to 2 hours and at 4° C for another 1 to 2 hours The mixture was centrifuged and the supernatant precipitated with ethanol and dissolved in sterile 2 M NaCl as before

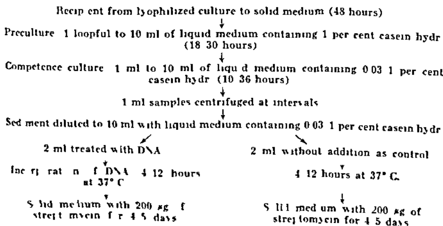
The concentration of the DNA solutions was determined by means of measuring their extinction at 260 mµ and 280 mµ in a Beckman DU spectrophotometer The stock solutions had concentrations of about *1 mg DNA per ml In* connections with the experiments the preparations were diluted to suitable concentrations

EXPERIMENTAL

In the transformation experiments the liquid medium mentioned above was used with the omission of the casamino acids and $CuSO_4$. When performing the experiments a solution of vitamin free casein hydrolysate was added.

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RESULTS

Initially the transformation experiments were performed with the liquid medium that was later used only for DNA preparation. Under these experimental conditions resistant mutants were obtained only rarely.

An increase in the number of transformed bacteria was in some cases

observed if the recipient cells were treated with 0.5–1 IE penicillin 4 hours before the addition of the DNA.

In the transformation system of *B. subtilis* Spizizen (4) found that Cu^{++}, even in very small concentrations strongly diminishes the frequency of transformation and that the concentration of casein hydrolysate is critical. The original *B. pertussis* medium contained $CuSO_4$ (0.5 mg per l) since Cu^{++} was supposed to be important for good growth. However $CuSO_4$ could be omitted from the original medium without any decrease in growth. When it was found that the transformations obtained after this elimination were more frequent, no further experiments with penicillin-treated cells were carried out.

Different amounts of casein hydrolysate in concentrations from 0.03 per cent to 1 per cent in the competence culture and in the DNA-treated sample gave different amounts of transformed bacteria. The experiments showed that with 1 per cent caseein hydrolysate, a concentration that provided good growth, no resistant cells occurred. However with 0.5 per cent or less casein hydrolysate transformed cells occurred. Even a culture with a concentration as low as 0.03 per cent casein hydrolysate permitted transformation, in spite of a very slow and limited growth. Table 1 illustrates experiments with different amounts of casein hydrolysate.

TABLE 1

Effect of Different Amounts of Casein Hydrolysate and of Varying DNA Incorporation Time. 4 Experiments with Recipient 44122/7 Treated with about 1 µg per ml of Purified DNA

Per cent casein hydrolysate	Resistant mutants per 0.5 ml of inoculum							
	Time between addition of DNA and plating in hours				Controls in hours			
	4	6	9	11	4	6	9	11
1	0	0	0	0	0	0	0	0
0.5	0	0	>400	>400*	0	0	0	0
0.25	5	51	>200	>200	0	0	0	0
0.12	0	39	70	200	0	0	0	0
0.06	0	2	20	100	0	0	0	0
0.03	0	10	2	5	0	0	0	0

*The very rough estimate depends on the difficulty ta spread the cells evenly as *B. pertussis* tends to grow as clusters in liquid media.

The pertussis bacteria were found to be competent for transformation during a rather long period, at least 18 to 20 hours. In rare instances competent bacteria were found in cultures about 10 hours old. From cultures of 20 to 27 hours age, however, the largest number and most constantly competent bacteria were found.

Only in rare instances and in a small number, resistant mutants were obtained if the samples were seeded on the solid medium containing streptomycin within 4 to 5 hours after the addition of DNA. In experi-

ments under similar conditions, resistant mutants were obtained if the interval was extended to 6 to 9 hours (Table 1).

Some of the resistant mutants which had grown on solid medium containing 200 μg of streptomycin per ml were tested for their degree of resistance They were all resistant to more than 5000 μg of streptomycin per ml on solid medium The resistance to streptomycin evidently was linked to another genetic marker, giving independence of starch since it was found that all transformeed cells tested (about 20) were independent of starch in the growth medium

DISCUSSION

Transformation experiments performed with bacteria such as pneumococci (9), *H influenzae* (3, 10), and *B subtilis* (13) show that in these systems competence develops rather quickly toward the end of the logarithmic growth phase and disappears soon after the stationary growh phase is reached The meningococci (6) constitute an exception as they are competent for a long time during their logarithmic growth phase

The division time for B pertussis exceeds by far that of other bacteria used in transformation experiments (it is about 3 hours in the medium used in these experiments) Therefore one can expect a longer duration and a less marked peak of competence than for other systems The results of the experiments show a competence time extending over at least 18 hours

In transformation experiments performed with pneumococci (8) and *H influenzae* (2) the phenotypic delay is about 2 hours If strepto mycin resistance is used as marker the cells are insusceptible to streptomycin after this time As the growth rate of *B pertussis* is considerably slower than that of these bacteria, one can expect a longer interval before resistance is definitely established This fact can explain that after 2 hours one can not demonstrate any resistant mutants at all, while after 4 hours only a few mutants are found An interval of 9 to 12 hours seems to be necessary for a high yield of resistant cells

The frequency of transformations has been low in most experiments but is nevertheless significant when seen against the fact that the spontaneous mutation rate of *B pertussis* toward streptomycin-resistance is very low According to *Alexander et al* (1) it is in the magnitude of one per 10^{10} to 10^{11} bacteria per ml

SUMMARY

A streptomycin sensitive strain of *B pertussis* has been transformed to streptomycin-resistance with DNA from a streptomycin-resistant mutant The streptomycin-resistance seems to be linked to independence of starch in the growth medium

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RESISTANCE TO METHICILLIN, ISOXAZOLYL PENICILLINS, AND CEPHALOTHIN IN *STAPHYLOCOCCUS AUREUS*

By

Knud Rieverts Eriksen and Ingrid Erichsen

Received 18 [illegible] 64

During the last few years a number of penicillinase resistant penicillins has become available for clinical use (7, 23). Methicillin introduced in 1960, and the more recently introduced isoxazolyl compounds oxa … sed clinically in Denmark since 1960

… (Ancillin®) and nafcillin have been available for clinical trials in the United States (2, 22, 26, 27). Recently a new series of semisynthetic compounds closely related to penicillin, the cephalosporins, has received much interest (1, 7, 23). One of these preparations, cephalothin, has been subjected to clinical investigation in U.S.A. (27).

For all these compounds the rationale for their use in infections caused by penicillin resistant staphylococci is their resistance against the action of staphylococcal penicillinase. Especially with methicillin clinical experience is very large and this penicillin is generally considered the drug of choice for treatment of severe infections with penicillinase-producing staphylococci.

However, it is known from laboratory experiments that penicillinase producing strains of *Staphylococcus aureus* can by subculturing in the presence of methicillin acquire an increased resistance to methicillin without loss of properties generally considered to be associated with virulence ([illegible], 11, 30). Such strains show an increased resistance also against other penicillinase-resistant penicillins and the cephalosporins (6, 7, 30).

Naturally occurring strains of *Staphylococcus aureus* resistant to methicillin and to the other penicillinase resistant penicillins including the cephalosporins have been described by a number of authors (5, 7,

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Semisynthetic penicillins were kindly supplied by *H. Lundbeck & Co. A/S*, cephalothin [illegible]



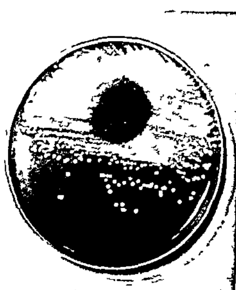


Fig 1 *Fig 2*

Fig 1 Methicillin sensitive *Staphylococcus aureus*
Fig 2 Methicillin resistant *Staphylococcus aureus*

8, 9, 10, 12, 17, 20, 21, 28, 29) since the first report by *Jevons* in 1961 (19) of methicillin-resistant strains isolated from clinical sources

In a previous paper (15), the isolation of methicillin-resistant strains of *Staphylococcus aureus* from two patients in a Danish hospital was reported In this paper the isolation of similar strains from eight additional patients is reported together with some experimental findings concerning the nature of methicillin resistance

MATERIAL AND METHODS

The methicillin resistant strains of *Staphylococcus aureus* reported in this paper have been found either among pure cultures forwarded from dr *Kirsten Rosendal* Statens Seruminstitut or they have been isolated from material sent to the Institute of General Pathology for bacteriological diagnosis

Testing for sensitivity to methicillin has been performed on all strains received or isolated in the laboratory since October 1960 Until the end of the year 1963 more than 14 000 strains isolated from clinical sources have been tested strains isolated from healthy nasal carriers among hospital staffs not being included in this number

Tests for methicillin sensitivity were made by the paper disc method described by *Jensen & Kjær (18)* The amount of methicillin used for each disc has been 50 µg The disc (20 mm in diameter) is removed after 30 minutes and the plate is inoculated with a platinum loop in such a way that one third of the area covered by the disc is more heavily seeded than the rest

By this method methicillin sensitive staphylococci after over night incubation at 37° C show inhibition zones at least 40 mm in diameter (Fig 1) With a methicillin resistant strain the inhibition zone is usually not more than 25 mm in diameter (Fig 2) This method has been used in all cases in which a rapid sensitivity determination was required *i e* before pure cultures of the staphylococci present in the specimen could be made

As a routine however the test has been performed on pure cultures isolated from single colonies From an overnight broth culture inoculation has been made in a streak at a right angle against the impression made by the edge of the removed paper disc

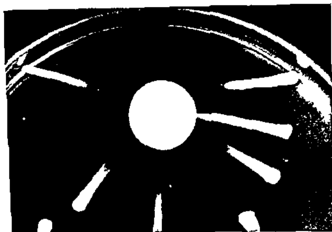


Fig 3

Streak plate w th ne methicill n resistant and five meth cill n sens tive strains of *Staphylococcus aureus* The or ginal paper d sc was rem ed bef re inoculation A new l s has l een appl ed l ecause the impression made by the or g nal d sc is not easily seen in a photo

W h this method the inh b tion zones for sensiti e strains are 10 mm or more f r resistant ones less than 5 mm (Fig 3) All strains found res stant l y one of

p a e t in an incul ator at 37 C an l read at the end of 24 and 48 hours f r visible gr wth

F r each experiment tl e numl ers of living staphylococcal cells in the cultures u ed as ino lum have l een estimate l by plating su table d lutions and count ng the numl ers f c l nies appearing after er night incul ation The numl ers have for all stra n use l l een 100 150 × 10 per ml

In exp riments with s l d media nutr ent agar was used The plates were 8 5 cm n d ameter The in cul m n a v lume f 0 1 ml was spread over the surface f the me l um w th a l nt *Laster r* p tte Col nies were counted after 24 and 48 h urs in ul at n at 37 C

Inact at n f the vari us c mp und was teste l in 5 ml l r th cultures in oculated w th unl lute l l roth culture as de cribed al ove After incul ation for var ng per l at 37 C the penic llinase effect was interrupte l by f ltration thr gh a S t f lter Tl e entire ult re was use l f r this purp se Thus in each experime t a n mber f singl cult ires was u e l The cultures were however pre pa e l n l a was tl a

n ne exper ment bein
n w l m l a l just been
h l l ng the ame c n e
en l f the experiment
f r a l t

D t rm nat n f drug act v ty in the Se t filtered cultures was made l y the agar p meth l lescr bed l *Vester l* l 31 As test rgani m *Staphyloc ccus aureus* FDA 209 P wa u ed

P n ll nase pr du t n has been dem nstrated by a mod fied C ts techn que

17 ... 72 2



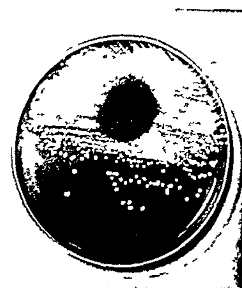


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Fig 2 Methicillin resistant *Staphylococcus aureus*

8, 9, 10, 12, 17, 20, 21, 28, 29) since the first report by *Jevons* in 1961 (19) of methicillin-resistant strains isolated from clinical sources

In a previous paper (15), the isolation of methicillin-resistant strains of *Staphylococcus aureus* from two patients in a Danish hospital was reported In this paper the isolation of similar strains from eight additional patients is reported together with some experimental findings concerning the nature of methicillin-resistance

MATERIAL AND METHODS

The methicillin resistant strains of *Staphylococcus aureus* reported in this paper have been found either among pure cultures forwarded from dr *Kirsten Rosendal* Statens Seruminstitut or they have been isolated from material sent to the Institute of General Pathology for bacteriological diagnosis

Testing for sensitivity to methicillin has been performed on all strains received or isolated in the laboratory since October 1960 Until the end of the year 1963 more than 14 000 strains isolated from clinical sources have been tested strains isolated from healthy nasal carriers among hospital staffs not being included in this number

Tests for methicillin sensitivity were made by the paper disc method described by *Jensen & Kjær (18)* The amount of methicillin used for each disc has been 50 µg The disc (20 mm in diameter) is removed after 30 minutes and the plate is inoculated with a platinum loop in such a way that one third of the area covered by the disc is more heavily seeded than the rest

By this method methicillin sensitive staphylococci after over night incubation at 37° C show inhibition zones at least 40 mm in diameter (Fig 1) With a methicillin resistant strain the inhibition zone is usually not more than 25 mm in diameter (Fig 2) This method has been used in all cases in which a rapid sensitivity determination was required i e before pure cultures of the staphylococci present in the specimen could be made

As a routine, however the test has been performed on pure cultures isolated from single colonies From an overnight broth culture inoculation has been made in a streak at a right angle against the impression made by the edge of the removed paper disc

TABLE 1

Sources of Methicillin Resistant Strains of Staphylococcus aureus

Case no	Hospital	Date of first isolation of the methicillin resistant strain	Material from which the strain was isolated	Sensitivity pattern	Phage type	Antibiotic treatment prior to first isolation of the methicillin-resistant strain
1	A	Aug 1962	sputum urine pus from abscess	PST	6/75/77	unknown
2	A	Aug 1962	tracheal aspirate	PST	6/75/77	no treatment
3	B	June 1963	pus from fistula colli and from tracheostomy	PSTI	47/54/77	penicillin chlortetracycline
4	C	July 1963	pus from post operative wound infection	PSTI	N T	penicillin streptomycin
5	D	Nov 1963	pus from lung abscess	PSTI	7/47/54/77	penicillin oxytetracycline streptomycin
6	I	Dec 1963	pus from post operative wound infection	PSTL	47/53/77	erythromycin chlortetracycline
7	D	Dec 1963	sputum	PSTE	47/54/77+	penicillin
8	D	Dec 1963	sputum	PSTE	7/47/53/54/77	penicillin
9	I	Dec 1963	pus from pleural empyema	PSTE	7/47/53/54/77/83A	penicillin streptomycin rolitetracycline
10	I	Dec 1963	pus from post operative wound infection	PSTE	7/47/54/77+	penicillin streptomycin

PSTI Resistant to penicillin streptomycin, tetracyclines and erythromycin
PST Resistant to penicillin streptomycin and tetracyclines

using agar containing 0.04 units/ml of penicillin G and as test organism a highly penicillin-sensitive strain of *B. subtilis* (14, 16).

Penicillinase-negative mutants were isolated from broth cultures kept for 2-3 weeks at room temperature. After subculturing on agar plates 50 separate colonies were tested for penicillinase production. From colonies which appeared negative at *Gots* plates broth cultures were made and tested once more for lack of penicillinase activity by overnight incubation in broth with 1 unit of penicillin G per ml.

Phage typing was performed in Statens Seruminstitut, Copenhagen.

The methicillin-resistant *Staphylococcus aureus* S. 5974 used in most experiments was isolated from case 1 (Table 1).

The methicillin sensitive strains of *Staphylococcus aureus* used in various experiments were:

326: a non penicillinase-producing strain fully sensitive to penicillin G, phage-type 80/81,

2719: a potent penicillinase-producer, phage type 80/81.

1634: a potent penicillinase producer with a high tendency to split off penicillinase-negative variants, phage type 80/81.

RESULTS

Methicillin-resistant strains of *Staphylococcus aureus* have been isolated from ten patients admitted to six different hospitals in Copenhagen. From most cases the resistant strain has been isolated on more than one occasion. Table 1 shows, for each patient, the sites from which the methicillin-resistant strain was isolated together with the date for its first isolation. The hospital to which the patient was admitted when the presence of methicillin-resistant staphylococci was first recognized is indicated by a capital.

Phage-types and sensitivity-patterns are shown in Table 1. All strains were resistant to penicillin, streptomycin, and tetracyclines and, with the exception of the strains isolated from cases 1 and 2, also to erythromycin. They were sensitive to chloramphenicol, neomycin, kanamycin, and fusidic acid.

One strain (case 4) was non-typable, all other strains belonged to phage group III. The strains from cases 1 and 2 appeared identical. The strains from the remaining seven cases showed identical or closely related phage-typing patterns, but were distinctly different from the strains isolated from cases 1 and 2.

Susceptibility to Methicillin, Oxacillin, Cloxacillin, Cephalothin, and Penicillin G

All strains found methicillin-resistant by the disc method have been tested in fluid medium against graded concentrations of methicillin, using an inoculum of 0,5 ml of an undiluted over-night broth culture. After 24 hours incubation at 37° C they all grow in 10 μg/ml. After 48 hours incubation all strains grow in 500, some even in 1000 μg/ml. However, no significant difference in sensitivity of various strains could be

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We are grateful to *Kirsten Rosendal* M.D. for typing a large number of strains.

d monstrated in one experiment a strain may grow in 1000 in another one only in 500 μg ml

Staphylococcus aureus S 5974 isolated from case 1 has been sub jected to further studies Its susceptibility against all four penicillins and cephalothin has been tested in fluid medium using various inoc ula and has been compared with that of the methicillin sensitive peni cillinase producing *Staphylococcus aureus* 2719

It is evident from the results of the experiments with the three peni cillinase resistant penicillins (Table 2) that the susceptibility of staphy lococcus S 5974 against all these compounds is significantly lower than that of staphylococcus 2719

The resistant strain shows however a rather significant inoculum size effect The susceptibility of the smallest inocula employed is in fact only insignificantly lower than that of the sensitive strain This phenomenon is most marked with methicillin and cloxacillin

In the case of methicillin the inoculum effect observed contrasts sharply to the findings with the sensitive strain which does not show any real inoculum effect at all However with oxacillin and cloxacillin also the sensitive strain exhibits a certain inoculum effect due to the fact that these penicillins are less resistant against penicillinase than is methicillin (6)

Also against cephalothin the susceptibility of the resistant strain is significantly lower than that of the sensitive strain (Table 3) The observed Also the sen the same degree as that

The susceptibility to penicillin G (Table 4) is likewise lower for the methicillin resistant strain than for the sensitive strain but because of the high penicillinase activity of the methicillin sensitive strain the difference between the two strains is not significant when comparing tul s seeded with very large inocula With the smallest inoculum used however the difference between the two strains is very significant the sensitivity of the resistant strain being 8 16 times lower than that of the sensitive one

With the same technique as used in the experiments described above two other strains 1709 and 1743 isolated from cases 3 and 4 respec tively have been tested against methicillin oxacillin and penicillin G The results of these experiments were virtually identical to those ob tained with staphylococcus S 5974

Inactivation of Methicillin and Oxacillin during Growth of the Methicillin Resistant Staphylococcus

In an attempt to elucidate the mechanism of the rather marked inoc ulum size-effect of the methicillin resistant strain subcultures were made from cultures grown in broth with 100 μg ml of methicillin These

TABLE 2

Susceptibility of a Methicillin Resistant and a Methicillin Sensitive Penicillinase Producing Staphylococcus aureus *to Methicillin Oxacillin and Cloxacillin*

	Methicillin resistant *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture					Methicillin sensitive *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture				
	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}
Methicillin Conc in µg/ml										
100	+ +	— —	—	— —	— —	— —	— —	— —	— —	— —
50	+ +	— +	—	—	— —	— —	— —	— —	— —	— —
25	+ +	+	—	—	— —	— —	— —	— —	— —	— —
10	+ +	— +	— +	— —	— —	— —	— —	— —	— —	— —
5	+ +	+ +	+ +	— +	— —	— —	— —	— —	— —	— —
2 5	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— —	— —	— —
1 25	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Oxacillin Conc in µg/ml										
1000		— —	— —	— —	— —	— —	— —	— —	— —	— —
100	+ +	— +	— —	— —	— —	— —	— —	— —	— —	— —
10	+ +	+ +	— +	— +	— —	+ +	— —	— —	— —	— —
5	+ +	+ +	+ +	— +	— —	+ +	— —	— —	— —	— —
2 5	+ +	+ +	+ +	+ +	— +	+ +	— —	— —	— —	— —
1 25	+ +	+ +	+ +	+ +	— +	+ +	— —	— —	— —	— —
0 625	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— +	— —	— —
0 312	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Cloxacillin Conc in µg/ml										
1000	— —	—	— —	— —	— —	— —	— —	— —	— —	— —
100	+ +	— +	— —	— —	— —	— —	— —	— —	— —	— —
10	+ +	+	— —	— —	— —	— +	— —	— —	— —	— —
5	+ +	— +	— +	— +	— —	— +	— —	— —	— —	— —
2 5	+ +	+ +	— +	— +	— —	+ +	— —	— —	— —	— —
1 25	+ +	+ +	+ +	— +	— —	+ +	— —	— —	— —	— —
0 625	+ +	+ +	+ +	— +	— —	+ +	— —	— —	— —	— —
0 312	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— +	— +
0 156	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

+ + growth after 24 hours incubation   + growth after 48 hours incubation   — — no growth

demonstrated in one experiment a strain may grow in 1000 in another one only in 500 μg ml

Staphylococcus aureus S 5974 isolated from case 1 has been subjected to further studies Its susceptibility against all four penicillins and cephalothin has been tested in fluid medium using various inocula and has been compared with that of the methicillin sensitive penicillinase producing *Staphylococcus aureus* 2719

It is evident from the results of the experiments with the three penicillinase resistant penicillins (Table 2) that the susceptibility of staphylococcus S 5974 against all these compounds is significantly lower than that of staphylococcus 2719

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Also against cephalothin the susceptibility of the resistant strain is significantly lower than that of the sensitive strain (Table 3) The same inoculum effect as with the penicillins is observed Also the sensitive strain shows an inoculum effect of nearly the same degree as that seen with oxacillin and cloxacillin

The susceptibility to penicillin G (Table 4) is likewise lower for the methicillin resistant strain than for the sensitive strain but because of the high penicillinase activity of the methicillin sensitive strain the difference between the two strains is not significant when comparing tubes seeded with very large inocula With the smallest inoculum used however the difference between the two strains is very significant the sensitivity of the resistant strain being 8 16 times lower than that of the sensitive one

With the same technique as used in the experiments described above two other strains 1799 and 1743 isolated from cases 3 and 4 respectively have been tested against methicillin oxacillin and penicillin G The results of these experiments were virtually identical to those obtained with staphylococcus S 5974

Inactivation of Methicillin and Oxacillin during Growth of the Methicillin Resistant Staphylococcus

In an attempt to elucidate the mechanism of the rather marked inoculum size effect of the methicillin resistant strain subcultures were made from cultures grown in broth with 100 μg ml of methicillin These

TABLE 3

Susceptibility of a Methicillin Resistant and a Methicillin Sensitive Penicillinase Producing Staphylococcus aureus *to Cephalothin*

	Methicillin resistant *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture					Methicillin sensitive *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture				
Cephalothin Conc in µg/ml	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}
100	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —
50	— +	-	— —	— —	— —	— —	— —	— —	— —	— —
25	+ +	— +	— —	— —	— —	— —	— —	— —	— —	— —
10	+ +	— +	— —	— —	— —	— +	— —	— —	— —	— —
5	+ +	— +	— —	— —	— —	+ +	— —	— —	— —	— —
2 5	+ +	— +	— +	— —	— —	+ +	— —	— —	— —	— —
1 25	+ +	+ +	— +	— —	— —	+ +	— —	— —	— —	— —
0 625	+ +	+ +	+ +	— +	— +	+ +	— —	— —	— —	— —
0 312	+ +	+ +	+ +	+ +	+ +	+ +	— +	— —	— —	— —
0 156	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— +	— +
0 078	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

+ + growth after 24 hours incubation — + growth after 48 hours incubation — — no growth

TABLE 4

Susceptibility of a Methicillin Resistant and a Methicillin Sensitive Penicillinase Producing Staphylococcus aureus *to Penicillin G*

	Methicillin resistant *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture					Methicillin sensitive *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture				
Penicillin G Conc in µg/ml	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}
10000	+ +	— —	— —	— —	— —	+ +	—	—	— —	— —
1000	+ +	— —	— —	— —	— —	+ +	— —	— —	— —	— —
100	+ +	+ +	— —	—	— —	+ +	— +	— —	— —	— —
10	+ +	+ +	— +	- —	— —	+ +	+ +	— —	- —	— —
5	+ +	+ +	+ +	+ +	— +	+ +	+ +	— —	— —	— —
2.5	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— —	— —	— —
1.25	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— +	— —	— —
0.625	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— —	— —
0.312	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

+ + growth after 24 hours incubation — + growth after 48 hours incubation — — no growth

TABLE 3

Susceptibility of a Methicillin Resistant and a Methicillin Sensitive Penicillinase Producing Staphylococcus aureus *to Cephalothin*

	Methicillin resistant *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture					Methicillin sensitive *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture				
Cephalothin Conc in µg/ml	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}
100	— —		— —	— —	— —	— —	— —	— —	— —	— —
50	- +	— —	— —	— —	— —	— —	— —	— —	— —	— —
25	+ +	— +	— —	— —	— —	— —	— —	— —	— —	— —
10	+ +	— +	— —	— —	— —	— +	— —	— —	— —	— —
5	+ +	- +	— —	— —	— —	+ +	— —	— —	— —	— —
2 5	+ +	— +	— +	— —	— —	+ +	— —	— —	— —	— —
1 25	+ +	+ +	— +	—	— —	+ +	— —	— —	— —	— —
0 625	+ +	+ +	+ +	— —	— —	+ +	— —	— —	— —	— —
0 312	+ +	+ +	+ +	— +	— +	+ +	— +	— —	— —	— —
0 156	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— +	— +
0 078	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

+ + growth after 24 hours incubation - + growth after 48 hours incubation — — no growth

TABLE 4

Susceptibility of a Methicillin Resistant and a Methicillin Sensitive Penicillinase Producing Staphylococcus aureus *to Penicillin G*

	Methicillin resistant *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture					Methicillin sensitive *Staph aureus* Initial number of staphylococci per ml as concentration of 24 hrs culture				
Penicillin G Conc in µg/ml	10 1	10 3	10 5	10 7	10 8	10 1	10 3	10 5	10 7	10 8
10000	+ +	— -		-	—	+ +	- —		— —	— —
1000	+ +	—			—	+ +	— —	—	— —	— —
100	+ +	+ +	— —	—		+ +	+		— —	— —
10	+ +	+ +	+	— —	—	+ +	+ +	— —	—	— —
5	+ +	+ +	+ +	+ +	— +	+ +	+ +	-	—	— —
2 5	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— —	— —	— —
1 25	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— +	— —	— —
0 625	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— —	— —
0 312	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

+ + growth after 24 hours incubation — + growth after 48 hours incubation — — no growth

subcultures were influenced by methicillin in exactly the same way as the original culture It thus appeared probable that the inoculum effect might be due to inactivation of methicillin (15)

That methicillin is actually inactivated during growth of the methicillin-resistant strain has been shown in experiments in which cultures of staphylococcus S 5974 in broth with respectively 10 and 100 μg ml of methicillin were tested for methicillin-activity after growth for various periods of time

In tubes with an initial concentration of 10 μg/ml inoculated with 0,5 ml of an undiluted culture the concentration of methicillin was after 10 hours incubation at 37° C 5, and after 18 hours 2 5 μg/ml

With an initial concentration of 100 μg/ml the activity after 24 hours was 10–25 and after 48 hours less than 2 μg/ml

Also oxacillin is inactivated during growth of the resistant strain In tubes with an initial concentration of 10 μg/ml the concentration after 6 hours incubation was less than 0 3 After 6 hours incubation of cultures with an initial concentration of 100 μg/ml the activity corresponded to 5 2 5 μg/ml and after 12 hours incubation the concentration was only 0 3 μg/ml

In all experiments of this kind tubes with uninoculated broth containing the same concentrations of drug as used in the inactivation test have been incubated and have been tested for activity after filtration through a *Seitz* filter No loss of activity has been observed

The methicillin-inactivating properties of all other methicillin resistant strains have been tested All strains inactivate methicillin to virtually the same degree

It thus appears as if the inoculum effect is, at least partly, a result of inactivation of methicillin

Relationship between Resistance to Methicillin and Production of Penicillinase

In order to examine further the relationship between methicillin resistance and the inactivation of methicillin the sensitivity of the resistant staphylococcus S 5974 has been compared to that of a penicillinase-negative strain derived from the original methicillin-resistant one This strain showed the same phage-type and sensitivity-pattern as the original strain S 5974

In broth the penicillinase-negative strain showed a significantly lower resistance to methicillin than did the original strain (Table 5) In tubes inoculated with 0 5 ml of an undiluted culture growth occurred after 24 hours in 10 μg ml and after 48 hours in 100 μg ml, while the original strain grew in 100 and 600 μg ml respectively With smaller inocula growth was seen in 1 25 μg ml, the original strain growing in 2 5–5 μg/ml

Thus, also with this penicillinase negative strain a certain inoculum

TABLE 5

Susceptibility of the Parent Strain and a Penicillinase Negative Mutant of a Methicillin Resistant Staphylococcus aureus *to Methicillin*

	Parent strain Initial number of staphylococci per ml as concentration of 24 hrs culture					Penicillinase negative mutant Initial number of staphylococci per ml as concentration of 24 hrs cultur				
Methicillin Conc in µg/ml	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}	10^{1}	10^{3}	10^{5}	10^{7}	10^{8}
800	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —
600	— +	— —	— —	— —	— —	— —	— —	— —	— —	— —
400	+	— —	— —	— —	— —	— —	— —	— —	— —	— —
200	— +	— —	— —	— —	— —	— —	— —	— —	— —	— —
100	+ +	— +	— —	— —	— —	— +	— —	— —	— —	— —
50	+ +	— +	— —	— —	— —	— +	— —	— —	— —	— —
25	+ +	— +	— —	— —	— —	— +	— +	— —	— —	— —
10	+ +	+ +	— +	— —	— —	+ +	— +	— —	— —	— —
5	+ +	+ +	+ +	— +	— +	+ +	— +	— —	— —	— —
2.5	+ +	+ +	+ +	+ +	+ +	+ +	+ +	— +	— —	— —
1.25	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

+ + growth after 24 hours incubation — + growth after 48 hours incubation — — no growth

effect was observed, but less marked than for the parent strain and the inhibitory concentrations were, with all inocula, significantly lower than for the original strain

This experiment has clearly shown that the original methicillin-resistant staphylococcus is significantly more resistant to methicillin than is the penicillinase-negative strain derived from it

The association between loss of penicillinase activity and increased susceptibility to methicillin is, perhaps, even better demonstrated by comparing the growth of the two strains on methicillin-containing agar plates

With an inoculum of 0 1 ml undiluted broth culture of the original strain confluent growth is obtained in 24 hours at concentrations up to 10 µg/ml At plates with 100 µg/ml about 200 very small colonies are seen after 24 hours After 1 or 2 days further incubation most of them have grown out to large, pigmented colonies with a distinct tendency to occur in clumps (Fig 4) The total number of colonies does usually not increase significantly above the number seen after 24 hours, except for a varying number of small colonies which may sometimes grow out around the larger colonies

With the penicillinase-negative strain, however, confluent growth is not seen at concentrations higher than 1 25 or 2 5 µg/ml At 10 µg/ml the number of colonies after 24 hours is about 100, all colonies being very small During the next days more colonies appear and after 3 days incubation the number is 200 or more At 100 µg/ml no growth is seen after 24 hours, after 48 hours about 25 very small colonies have grown out and after 3 days incubation the number of colonies is about 100 The colonies do not tend to be clumped together, as was the case with the original strain After 3 days incubation the colonies, which first appeared, have grown out to large, pigmented ones The youngest colonies are small and unpigmented, and all possible intermediate forms between these and the large ones are seen (Fig 5)

The growth of the penicillinase-negative strain on agar with 100 µg/ml of methicillin is, thus, significantly slower than that of the original strain and the appearance of colonies is much more similar to that of penicillin-resistant colonies grown out on penicillin-containing agar plates seeded with a large inoculum of a penicillin-sensitive staphylococcus

When using, for comparison, the highest concentrations permitting confluent growth, the penicillinase-negative strain shows a susceptibility to methicillin 4–8 times higher than that of the original strain

Also with an inoculum of 0 1 ml of a 10^{-6} dilution of a broth culture (100–150 bacteria) the difference between the two strains is very significant

In Table 6 the minimal inhibitory concentrations as measured by this method after incubation for 24 and 48 hours, respectively, are given For comparison the corresponding values for two methicillin-sensitive strains are also given For each strain the numbers of colonies



Fig 4 Fig 5

Fig 4 Plate with nutrient agar containing 100 µg/ml of methicillin inoculated with 0 1 ml of broth culture of the methicillin resistant *Staphylococcus aureus* S 5974 Incubated for 72 hours at 37° C

Fig 5 Plate with nutrient agar containing 100 µg/ml of methicillin inoculated with 0 1 ml of broth culture of a penicillinase negative strain derived from *Staphylococcus aureus* S 5974 Incubated for 72 hours at 37° C

on all concentrations permitting growth are virtually identical all cells in the inoculum growing out to colonies The colonies appearing after 24 hours on the highest concentration are often small and unpigmented and growth may as indicated in the table be visible first after 48 hours incubation After 72 hours however the colonies on all concentrations for all strains appear as typical pigmented staphylococcal colonies

Thus it appears also from this experiment that the penicillinase negative strain is more sensitive to methicillin than the original methicillin resistant strain

The experimental results given in Table 6 have further shown that the growth of the penicillinase-negative strain is inhibited by a concentration of methicillin only double as high as the concentration which inhibits the growth of a methicillin sensitive staphylococcus

Several other penicillinase negative strains (all of identical phage type and sensitivity pattern) derived from *Staphylococcus aureus* S 5974 have been tested with the same technique The results have always been virtually the same as those given in Table 6

Susceptibility of the Methicillin Resistant Staphylococcus to Penicillin G

From experiments in which the susceptibility to penicillin G was estimated in fluid medium it appeared probable that the methicillin resistant staphylococcus S 5974 had an inherent resistance to penicillin (Table 4) However no certain conclusions can be drawn from

experiments of that kind because inactivation by penicillinase may play a rôle even when the inoculum is very small. With the isolation of penicillinase-negative strains, however, a possibility was offered for proving the existence of the "inherent" *penicillin-resistance suggested*.

Penicillinase-negative strains derived from a penicillinase-producing *Staphylococcus aureus* have usually been found fully sensitive to penicillin (4). Therefore, the strains have been compared with a penicillin-sensitive staphylococcus and with a penicillinase-negative strain de*rived from a methicillin-sensitive penicillinase-producing staphylococ*cus. Unfortunately, no penicillinase-negative strains could be isolated from staphylococcus 2719 used in other experiments. Staphylococcus 1634 used in this experiment is, as staphylococcus 2719, a potent penicillinase-producer and it shows the same degree of resistance to penicillin *G*.

TABLE 6

Susceptibility to Methicillin of a Methicillin Resistant Staphylococcus aureus, *a Penicillinase-Negative Strain derived from it, and of two Methicillin Sensitive Strains of* Staph. aureus *Estimated on Solid Medium Inoculated with 0.1 ml of a 10[illegible] Dilution of a 18 Hours Broth Culture (100-150 Bacteria)*

	Minimal inhibitory concentrations of methicillin in μg/ml	
	24 hrs incubation	48 hrs incubation
Methicillin resistant *Staph. aureus* S. 5974	10	10
Penicillinase negative strain (No 1) derived from *Staph. aureus* S. 5974	2.5	5
Methicillin sensitive penicillinase producing *Staph. aureus* 2719	1.25	2.5
Methicillin sensitive non penicillinase-producing *Staph. aureus* 326	1.25	1.25

Colonies counted after 24 and 48 hours incubation at 37° C.

TABLE 7

Susceptibility to Penicillin G of a Penicillin-Sensitive Staphylococcus aureus *and of Penicillinase Negative Strains Derived from a Methicillin Resistant and from a Methicillin Sensitive* Staphylococcus aureus *Estimated on Solid Medium Inoculated with 0.1 ml of a 10[illegible] Dilution of a 18 Hours Broth Culture (100-150 Bacteria)*

	Minimal inhibitory concentrations of penicillin G in μg/ml	
	24 hrs incubation	48 hrs incubation
Penicillinase negative strains derived from methicillin resistant *Staph. aureus* S. 5974 — No 1	0.08	0.156
— No 2	0.08	0.156
— No 3	0.156	0.312
Penicillinase negative strain derived from methicillin sensitive *Staph. aureus* 1634	0.02	0.02
Penicillin sensitive *Staph. aureus* 326	0.02	0.02

Colonies counted after 24 and 48 hours incubation at 37° C.

The results of an experiment, in which the susceptibility to penicillin G of these strains have been determined on solid media, are given in Table 7 As in the experiment with methicillin (Table 6) all cells in the inoculum give rise to colonies, if growth occurs at all

This experiment has shown that the penicillinase-negative strain derived from the methicillin sensitive, penicillinase-producing staphylococcus is, as might be expected, fully sensitive to penicillin G The three penicillinase negative strains derived from staphylococcus S 5974, however, are 4–8 times or, if the susceptibility is estimated from the growth after 48 hours incubation, 8 16 times more resistant to penicillin G than the sensitive staphylococcus

DISCUSSION

The methicillin resistant strains of *Staphylococcus aureus* described in this paper appear to be nearly related to the naturally occurring methicillin-resistant strains described by other authors Except for the strain isolated from case 4, which was non-typable, all have belonged to phage group III and the phage-typing patterns were very similar to those of the majority of other reported strains Also the sensitivity-patterns have been closely related to those reported by other authors, all strains hitherto reported being resistant to streptomycin and tetracyclines, many also to erythromycin (6, 7, 10, 20)

As to the degree of resistance to methicillin the Danish strains are obviously, closely related to the "Carshalton-strain" described by *Stewart & Holt* (29) Staphylococcus S 5974, which has been subjected to closer studies, appears also in respect to inoculum size effect, to resistance against the isoxazolyl penicillins and to inactivation of these penicillins to behave in the same manner as the "Carshalton-strain" Only regarding the inactivation of methicillin are the results obtained with the Danish strains not in agreement with the results of these authors who did not [illegible]
how [illegible]
sent [illegible]

In [illegible] resistant staphylococci have subsequently been observed by *Ayliffe & Barber* (3) working with, among other strains S 5974 used in the experiments reported above The findings by *Stewart & Holt* concerning inactivation of cloxacillin have been confirmed by *Knox & Smith* (25)

Stewart & Holt (29) emphasized that their "Carshalton-strains" differed in several ways from the first, naturally resistant strains reported by *Jevons* (19), the main difference being that the strains of *Jevons* contained a mixed population of cells the majority of which were sensitive to methicillin

However, *Jevons et al* (20) do not emphasize this difference, despite

experiments of that kind because inactivation by penicillinase may play a rôle even when the inoculum is very small With the isolation of penicillinase negative strains, however, a possibility was offered for proving the existence of the "inherent" penicillin-resistance suggested

Penicillinase negative strains derived from a penicillinase producing *Staphylococcus aureus* have usually been found fully sensitive to penicillin (4) Therefore, the strains have been compared with a penicillin-sensitive staphylococcus and with a penicillinase-negative strain derived from a methicillin sensitive penicillinase-producing staphylococcus Unfortunately, no penicillinase-negative strains could be isolated from staphylococcus 2719 used in other experiments Staphylococcus 1634 used in this experiment is, as staphylococcus 2719, a potent penicillinase-producer and it shows the same degree of resistance to penicillin G

TABLE 6

Susceptibility to Methicillin of a Methicillin Resistant Staphylococcus aureus *a Penicillinase Negative Strain derived from it and of two Methicillin Sensitive Strains of* Staph aureus *Estimated on Solid Medium Inoculated with 0 1 ml of a 10⁻⁴ Dilution of a 18 Hours Broth Culture (100 150 Bacteria)*

	Minimal inhibitory concentrations of methicillin in µg ml	
	24 hrs incubation	48 hrs incubation
Methicillin resistant *Staph aureus* S 5974	10	10
Penicillinase negative strain (No 1) derived from *Staph aureus* S 5974	2 5	5
Methicillin sensitive penicillinase-producing *Staph aureus* 2719	1 25	2 5
Methicillin sensitive non penicillinase producing *Staph aureus* 326	1 25	1 25

Colonies counted after 24 and 48 hours incubation at 37° C

TABLE 7

Susceptibility to Penicillin G of a Penicillin Sensitive Staphylococcus aureus *and of Penicillinase Negative Strains Derived from a Methicillin Resistant and from a Methicillin Sensitive* Staphylococcus aureus *Estimated on Solid Medium Inoculated with 0 1 ml of a 10⁻⁴ Dilution of a 18 Hours Broth Culture (100 150 Bacteria)*

		Minimal inhibitory concentrations of penicillin G in µg ml	
		24 hrs incubation	48 hrs incubation
Penicillinase negative strains derived from methicillin resistant *Staph aureus* S 5974	No 1	0 08	0 156
	No 2	0 08	0 156
	No 3	0 156	0 312
Penicillinase negative strain derived from methicillin sensitive *Staph aureus* 1634		0 02	0 02
Penicillin sensitive *Staph aureus* 326		0 02	0 02

Colonies counted after 24 and 48 hours incubation at 37° C

to be readjusted in as much as the inactivation observed must necessarily cause some inoculum effect

Experiments reported above, with penicillinase negative strains derived from a methicillin resistant staphylococcus have shown such strains to be significantly more sensitive to methicillin than the original penicillinase producing strain

Penicillinase negative strains derived from penicillinase producing staphylococci usually possess all characteristics of the parent strain except penicillinase production There is no reason for questioning that this was true also for the strains used in these experiments They were all of the same phage type as the original strain and growth rate and appearance of colonies were likewise unchanged In our opinion it is therefore justified to conclude from these experiments that methicillin resistance of the type described in this paper, and the inoculum effect characteristic of this kind of resistance, is at least partly a result of methicillin inactivation

Certainly also with the penicillinase negative strains some inoculum effect was observed Rather than concluding that such strains represent mixed cultures this phenomenon may perhaps better be explained as the results of a very great tendency for resistant cells to arise during incubation on methicillin containing medium

That the appearance of colonies on methicillin containing agar inoculated with a penicillinase negative strain actually is very much like that of colonies grown out on penicillin containing agar inoculated with a fully sensitive staphylococcus appears evident when Fig 5 in the present paper is compared with the photo published in *Eriksen's* paper on the mode of origin of penicillin resistant staphylococci (13) The somewhat different appearance of growth on methicillin plates inoculated with the original methicillin resistant strain especially the more rapid development of colonies which tend to be clumped together may easily be explained as the result of a superimposed effect of penicillinase

Comparative investigations of penicillinase negative strains and the parent strain are continuing One problem to be investigated is that of the sensitivity to methicillin of staphylococci growing out on methicillin containing medium Preliminary experiments have shown that it is possible to select from a penicillinase negative strain after one passage on agar with 100 μg methicillin per ml coagulase positive staphylococci of unchanged phage typing pattern which grow without any significant inoculum effect in broth with 1000 μg ml of methicillin

Unfortunately we have not yet succeeded in selecting penicillinase negative mutants from other strains of methicillin resistant staphylococci Therefore our conclusions must be taken with some caution being subject to the reservations that they were drawn from experiments with one single strain of *Staphylococcus aureus*

With the possible exception of case 1 in which no information about

the fact that many of the methicillin-resistant strains described in their paper were actually those described by *Stewart & Holt* and others were received from the hospital in which the strains reported by *Jevons* in 1961 were isolated. They only mention that most resistant cultures grew continuously up to the methicillin-disc used in their plates and that a few, which contained a relatively small proportion of resistant individuals, showed only discrete colonies near the disc.

As a given strain may, undoubtedly, from day to day, show minor variations in its resistance to methicillin it seems unwarranted to draw any conclusions from differences of this kind. It appears more relevant to emphasize the great similarities between most clinically occurring methicillin-resistant strains hitherto described.

In fact, the experiments of *Knox & Smith* (24) with one of the strains described by *Jevons* in 1961 indicate a close similarity between this strain and the strains described in the present paper. Using a very small inoculum (about 10 bacteria) these authors found that the minimum inhibitory concentration of methicillin was four times greater for the resistant strain than for a methicillin sensitive penicillinase-producing strain, and they further found evidence of an "inherent" resistance to penicillin G. It is likewise worth mentioning that the strain 16137/1000 which by *Knox & Smith* (25) now is found to inactivate cloxacillin and methicillin was found to cause no inactivation.

A characteristic of naturally occurring methicillin resistant staphylococci, mentioned by several authors, is the marked inoculum-size-effect (6). Also the strains described in this paper have shown a significant inoculum effect. This is evident from the experiments carried out with staphylococcus S. 5974, experiments which have been repeated with two other strains with virtually the same results as those reported in Table 2 and 3.

The somewhat irregular shape of the inhibition zones shown by resistant strains (Fig. 2) is a clear illustration of the inoculum effect, the diameter of the zone being smallest in the most heavily seeded part of the field. In the streak method (Fig. 3) the inoculum used has been proportionately very large, and the resistant strains are best recognized by this method. In fact, if the inoculum is too small, methicillin-resistance may not be recognized at all. Ignorance of this is probably the cause of the deplorable fact that some methicillin resistant strains described in this paper have been reported by other laboratories as being fully sensitive to methicillin.

It has, so far, been generally agreed that the inoculum effect is not due to any inactivation of methicillin and that this effect is adequately accounted for by assuming that a culture of a methicillin-resistant staphylococcus contains a mixed population of cells of which only a minority is highly resistant (6, 24).

After the demonstration of a significant inactivation of methicillin during growth of a methicillin resistant staphylococcus this view has

in Denmark, of methicillin resistant strains of *Staphylococcus aureus* is far from being negligeable The information given in the table suggest that the occurrence of these strains is partly a result of cross-infection and further epidemiological analysis has shown that the strains isolated from six cases in 1963 were, presumably, of common origin

Case 5, from which two other patients (cases 7 and 8) in hospital D were cross infected, had been transferred to this hospital from a department for general surgery and were infected already while staying there Case 3 had obviously acquired the infection in this department too The strains from cases 9 and 10 were found among a collection of strains, kindly sent to us from dr Kirsten Rosendal They were isolated from specimens received from the same surgical department (hospital F) in which cases 3 and 5 acquired their infections

After the completion of the present paper an investigation has been commenced in this department Four nurses were found to carry the methicillin-resistant staphylococcus in their noses and from six patients with postoperative wound infections the same strain was isolated from pus It is noteworthy that penicillinase resistant penicillins have been used in this department only in exceptional cases It appears quite evident that the spread of the resistant strain in this department had no relationship whatever to the use of methicillin or other new penicillins Details about the results of these investigations will be published elsewhere

SUMMARY

Methicillin-resistant strains of *Staphylococcus aureus* have been isolated from ten patients admitted to six different hospitals in Copenhagen All strains were resistant to penicillin, streptomycin, and tetracyclines the majority also to erythromycin One strain was non-typable, all other strains belonged to phage group III

All strains were resistant not only to methicillin but also to oxacillin, cloxacillin and cephalothin A marked inoculum-size-effect was observed With a large inoculum all strains grew in broth containing 500 or 1000 µg ml of methicillin In fluid medium a significant [illegible]
of [illegible]

ne[illegible] [illegible] Comparative experiments have shown such strains to be significantly less resistant to methicillin than the parent penicillinase-producing strain

The penicillinase negative strains had an "inherent" [illegible]

[illegible] of the "naturally occurring" type are actually, strains of *Staphylococcus aureus* characterized

previous treatment has been available, no patient in the material reported in this paper was treated with methicillin or with other penicillinase-resistant penicillins Most strains of methicillin-resistant staphylococci reported by other authors have likewise been isolated from patients not treated with the new penicillins (10, 29) Actually, the first strains reported by *Jevons* (19) were isolated so soon after the introduction of methicillin and under such circumstances that it seems very probable that their occurrence had no relation whatever to the use of methicillin Interesting is also the report by *Cetin & Ang* (9) on methicillin-resistant strains of *Staphylococcus aureus* isolated from patients in Turkey before methicillin had become available in that country

It is, therefore, generally agreed that methicillin-resistant strains of *Staphylococcus aureus* isolated from clinical sources are "naturally resistant", but their ultimate mode of origin remains obscure (6)

It was shown in experiments reported above that penicillinase-negative strains derived from a methicillin-resistant *Staphylococcus aureus* were significantly less sensitive to penicillin G than other penicillinase-negative staphylococci Thus, this staphylococcus is penicillin-resistant not only as a result of its production of penicillinase, but it has also an "inherent" resistance to penicillin G

Strains with these properties may very well have existed before the introduction of methicillin, in as much as the existence of this kind of penicillin-resistance would have been extremely difficult, if not impossible, to demonstrate, at least in the routine laboratory where large numbers of staphylococci are tested

Perhaps, it is justified to consider naturally occurring methicillin-resistant staphylococci as strains of *Staphylococcus aureus* characterized by high penicillinase-production and a certain degree of "inherent" resistance to penicillin That such strains may occur without any relationship to the use of methicillin would not be surprising

The incidence of methicillin-resistant strains of *Staphylococcus aureus* is, undoubtedly, increasing *Jevons et al* (20) found among 27,479 cultures 102 resistant strains giving an incidence of 0 37 per cent, but throughout the period January 1961 to September 1962 the incidence rose from less than 0 25 to 0 8 per cent *Barber & Waterworth* (7) found that 3 per cent of the strains isolated from lesions in surgical patients were resistant Endemic spread of methicillin-resistant staphylococci in a children's hospital has been reported by *Stewart & Holt* (29)

Chabbert & Baudens (10) found ten resistant strains among 82 strains received from February 1961 to February 1962 This very high incidence is possibly due to the fact that their material consisted of strains isolated from patients whose infections for various reasons did not respond to antibacterial chemotherapy However, only one of their strains was isolated from a case treated with methicillin

The findings summarized in Table 1 indicate that the incidence, also

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by high penicillinase-production associated with an "inherent" resistance to penicillin G

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MATERIALS AND METHODS

Normal Human Sera

Sera from 187 unrelated adult healthy individuals from an earlier described material (*Berg* 1963) were used for association test Sera from selected families of an earlier described family material (*Berg & Mohr 1963*) were used for linkage study

Anti Ag(a) Serum

A sample of anti Ag(a) serum (C de B) was kindly provided by Dr B S Blumberg of the National Institutes of Health Bethesda Maryland This is the original serum used by Allison and Blumberg when they detected the Ag system

Anti Lp(a) Serum

Rabbit serum K 3 absorbed 2 5 with human serum of type Lp(a—) was used (see *Berg* 1963)

Petri Dishes

ANUMBRA dishes 7 cm in diameter were used

Glass Slides

Washed and polished 5 × 5 cm glass slides (Menzel Glaser) were used

Agar

For the Lp tests Difco Bacto Agar (Control No 452925) was used (Difco Laboratories Detroit 1 Mich)

For the Ag tests Rheinagar (Behringwerke AG Marburg Op Nr 141) was used The only reason for using different sorts of agar for the two systems was that one was more easily available than the other at the time of the investigations For the Lp tests the first agar is just as good as the second and there is no reason to believe that this is not so even for the Ag tests

Double Diffusion Tests in Agar Gel

For the Lp tests the agar gel was prepared in the Petri dishes as described elsewhere (Berg 1963) Six peripheral wells were cut around one central well the dia [illegible]

[illegible] chamber at 37° C Readings of precipitates were made after 2 days against a dark background by oblique illumination from below Visible precipitate was registrated as positive reaction no visible precipitate as negative reaction

For the Ag tests and all other experiments which included the anti Ag(a) serum the agar gel double diffusion experiments were performed on 5 × 5 cm glass slides to save anti Ag(a) [illegible]
layer The gel cont [illegible]
cent (v/v) 1/15 M [illegible]
On each slide 6 pe [illegible]
distances between [illegible]
serum was place l [illegible]
and the precipitates were read after 2 days in the same manner as described for the Petri dishes used for the Lp tests

Photographic Registrations

Photographic registrations of gel diffusion tests were made with a Leica 35 mm camera on Agepe Agfa film

Staining

The precipitates in the agar gel double diffusion tests were stained according to Uriel (1960) using either Oil Red O as a lipid stain or Amido Black 10 B as a protein stain (both from G T Gurr Ltd London)

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COMPARATIVE STUDIES ON THE Lp AND Ag SERUM TYPE SYSTEMS

By

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Received 8 V 64

During recent years two different genetic systems within human serum β-lipoprotein have been described The Ag system was described by *Allison & Blumberg* (1961). The authors demonstrated antigenic differences in human sera by means of an antibody in the serum from a patient who had received multiple transfusions, with Ouchterlony's agar gel double diffusion technique (*Ouchterlony* 1958). The antigen of the Ag system was shown to be β-lipoprotein (*Blumberg, Dray & Robinson* 1962)

The Lp system was discovered by *Berg* (1963) Using the same test technique, but with a heteroantiserum, he found antigenic differences in human serum β-lipoprotein The anti-Lp(a)-serum was obtained by intravenous immunization of rabbits with isolated human β-lipoprotein from one single donor A subsequent absorption procedure was made for the demonstration of the type specificity of the antiserum Later studies have shown, however, that even whole human serum can be used for immunization (These experiments will be described in another paper)

The genetics of the Lp system was studied in a material consisting of 94 Norwegian families with 305 children (*Berg & Mohr* 1963), and association and linkage studies were performed with the same material (*Mohr & Berg* 1963) Twenty-three normal human sera of known types within the Lp system were tested for the Ag(a) factor by Dr B S Blumberg of the National Institutes of Health, Bethesda, Maryland, and as far as these data extended, no interrelationship was found between the Ag(a) and the Lp(a) factors (see *Berg* 1963)

The purpose of the present paper is to present the results of more extensive comparative studies on the Lp and Ag serum type systems The data presented are obtained from immunological studies, from association test in a material consisting of unrelated individuals, and from linkage test in an investigation of informative families

intrabasin absorption technique At first 15 μl of the human serum for absorption was placed in one well When this serum had diffused into the surrounding agar leaving the well empty 15 μl of the anti serum was placed in the same well At the same time the human sera against which the absorbed antisera were to be tested were placed in the other wells The distribution of the different sera and the results obtained are shown in Fig 2

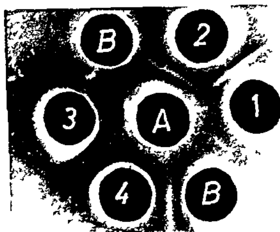


Fig 2

Agar gel slide showing the results of intrabasin absorption experiments with anti Ag(a) and anti Lp(a) sera (see text)

The reagents (15 μl of each) were
- A Human serum of type Lp(a) Ag(a+)
- B Human serum of type Lp(a+) Ag(a)
- 1 Anti Ag(a) serum absorbed with human serum of type Lp(a—) Ag(a+)
- 2 Anti Ag(a) serum absorbed with human serum of type Lp(a+) Ag(a—)
- 3 Anti Lp(a) serum absorbed with human serum of type Lp(a+) Ag(a—)
- 4 Anti Lp(a) serum absorbed with human serum of type Lp(a—) Ag(a+)

Note that type Lp(a+) Ag(a—) human serum has no ne

It was demonstrated that a normal human serum of type Lp(a+) Ag(a—) was unable to absorb the anti Ag(a) antibody from the anti Ag(a) serum whereas a human serum of type Lp(a—) Ag(a+) absorbed the anti Ag(a) antibody completely It was further demonstrated that a human serum of type Lp(a—) Ag(a+) was unable to absorb the anti Lp(a) antibody from the anti Lp(a) serum while a human serum of type Lp(a+) Ag(a—) absorbed all anti Lp(a) activity These experiments seem to confirm that the Ag(a) and the Lp(a) factors are antigenically unrelated

EXPERIMENTS AND RESULTS

Immunological Studies on the Lp(a) and Ag(a) Factors

Using the anti-Lp(a)- and anti-Ag(a)-sera in agar gel double diffusion tests, human sera of all 4 possible reaction types were found Thus some sera were found which were negative and others which were positive against both antisera, as well as sera of the reaction patterns Lp(a+) Ag(a—) and Lp(a—) Ag(a+) (See Fig 1)

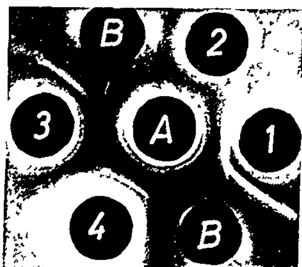


Fig 1

Agar gel slide demonstrating reactions between normal human sera (wells 1-4) and anti Lp(a)-serum (wells B) and anti-Ag(a)-serum (well A) The precipitation pattern shows that the human sera are of the following phenotypes

well 1 Lp(a+) Ag(a+)
well 2 Lp(a—) Ag(a—)
well 3 Lp(a+) Ag(a—)
well 4 Lp(a—) Ag(a+)

Note reaction of non identity between the Lp(a)- and Ag(a) precipitates near well 1

When human serum containing both the Ag(a) and the Lp(a) factors was tested simultaneously against anti-Lp(a)-serum and anti-Ag(a)-serum, *a reaction of non-identity developed between the two* precipitates (see Fig 1) The reaction of non-identity indicated that the Ag(a) and the Lp(a) factors are antigenically different

The following absorption experiment was performed in order to investigate further the apparent difference between the Ag(a) and Lp(a) antigens

Anti-Ag(a) serum was absorbed with a human serum of type Lp(a+) Ag(a—), and, for control, with a serum of type *Lp(a—) Ag(a+)* Likewise an anti-Lp(a)-serum was absorbed with a human serum of type Lp(a—) Ag(a+), and, for control, with a human serum of type Lp(a+) Ag(a—) To save the anti Ag(a)-serum, these absorption experiments were performed on 5 × 5 cm agar slides, using an

Lp(a+) Ag(a+) The antisera were placed such that they at the same time provided a control that the precipitation had really taken place in antibody excess The arrangement on the agar slide and the result obtained is shown in Fig 3

When human serum of type Lp(a+) Ag(a+) was precipitated with excess of anti Lp(a) serum so that the human serum no longer reacted with anti Lp(a) serum in the neighbouring well the human serum still reacted strongly with anti Ag(a) serum in another neighbouring well It was also found that when the human serum of type Lp(a+) Ag(a+) was precipitated with excess of anti Ag(a) serum so that it no longer reacted with anti Ag(a) serum it still reacted positively with anti Lp(a) serum (See Fig 3)

This experiment seems to prove that the Lp(a) and Ag(a) factors refer to different β lipoprotein molecules

Test for Association

For the association test sera from 187 unrelated adult Norwegians already tested in the Lp system were tested with anti Ag(a) serum Of these sera 62 (33.16 per cent) were of type Lp(a+) and 125 (66.84 per cent) of type Lp(a—) whereas 72 (38.50 per cent) were of type Ag(a+) and 115 (61.50 per cent) of type Ag(a—)

The distribution of the individuals in this material with respect to the Lp(a) and Ag(a) factors is shown in Table 1

TABLE 1

Distribution of 187 Unrelated Individuals with Respect to their Lp and Ag Types

	Ag(a+)	Ag(a−)	Total
Lp(a+)	28	34	62
Lp(a−)	44	81	125
Total	72	115	187

$\chi^2 = 1.3415$ 1 D F $0.20 < p < 0.30$

(χ^2 for this table was calculated as devised by *Bradford Hill* (1961) p 172)

The result of the χ^2 test makes an association between the Lp(a) and Ag(a) factors highly improbable

Test for Linkage

For linkage test the method devised by *Race & Sanger* (1962) using "double back cross" families was applied For this test one parent must be heterozygous for both the genes being investigated As antisera are available against only one factor within each of the two systems under study the only way to select heterozygous parents is for each system to select the families of positive × negative parents with at least one

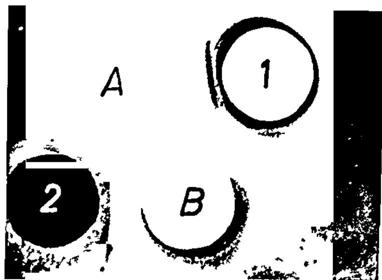


Fig 3
Tests in agar gel of human serum of type Lp(a+) Ag(a+) after precipitation with excess of anti-Ag(a)-serum and anti Lp(a)-serum respectively (see text)
The reagents (15 μl of each) were
1 Human serum of type Lp(a+) Ag(a+) and anti-Ag(a)-serum
2 Human serum of type Lp(a+) Ag(a+) and anti-Lp(a) serum
A Anti-Lp(a)-serum
B Anti-Ag(a)-serum
The precipitation pattern shows that human serum of type Lp(a+) Ag(a+) still reacted with anti-Lp(a)-serum after precipitation with excess of anti-Ag(a)-serum, and with anti-Ag(a)-serum after precipitation with excess of anti Lp(a)-serum This indicates that the Lp(a) and Ag(a) factors refer to different β lipoprotein molecules

Attempt to Find whether the Lp(a) and Ag(a) Properties Belong to the Same Protein Molecules

To investigate whether the Lp(a) and Ag(a) factors are located on the same lipoprotein molecules in persons possessing both factors, the following experiment was performed on a 5 × 5 cm agar slide

A human serum of type Lp(a+) Ag(a+) was precipitated with anti-Lp(a)-serum in antibody excess, and another sample of the same human serum with excess of anti-Ag(a)-serum The anti-Lp(a)-serum used in this experiment was specifically absorbed with a Lp(a—) Ag(a—) human serum so that no additional amount of Ag(a) factor should be introduced through the human serum used for absorption of the anti-Lp(a)-serum The anti-Ag(a)-serum was found to be of type Lp(a—)

At first, anti Ag(a)-serum was introduced into one well, and anti-Lp(a)-serum into another well, and the slide was placed in a moist chamber while the sera were diffusing into the agar gel After some two hours the wells were completely empty, and now the human serum of type Lp(a+) Ag(a+) was placed in both the wells, and at the same time anti-Lp(a)-serum and anti-Ag(a)-serum were placed in two other wells to test the effect of the precipitation on the human serum of type

able (*Berg & Mohr* 1963), the informative families were selected in this way

Of the total of 41 Lp(a+) × Lp(a—) matings, 28 families had at least one Lp(a—) child The parents of these 28 families were tested with respect to the Ag(a) factor The children of the families in which one parent was Lp(a+) Ag(a+), and the other Lp(a—) Ag(a—), were tested with respect to the Ag(a) factor Of 6 such families 4 families were found in which there was at least one Ag(a—) child Because of the small chance to find double heterozygous individuals when antisera are available against only one factor within each of the two systems, the number of families informative for linkage studies, had to be small The distribution of the Lp and Ag types in the four families informative as to possible linkage between the Lp(a) and the Ag(a) factors, is shown in Fig 4

Using the table of *Race & Sanger* (1962, p 417), the linkage score, λ, and information', γ, for each family, and the Σ (λ) and Σ (γ) for the material of four families were found (Table 2)

TABLE 2

Linkage Scores (λ) and Information (γ) for 4 "Double Back Cross" Families with Respect to the Ag and Lp Systems

Family No	Number of children in family	Ratio*	Linkage score λ	Information γ
1	3	2 1	—1	3
2	4	2 2	—2	6
3	3	2 1	—1	3
4	3	2 1	—1	3
			Σ (λ) = —5	Σ (γ) = 15

* ratio = the ratio of children that would do for linkage to those that would then have to be recombinants (see *Race & Sanger* (1962) p 417)

If there is linkage the Σ (λ) keeps on being positive as the data grow (*Race & Sanger* 1962) According to *Race & Sanger* (1962), the existence of linkage has been demonstrated at the 1 in 20 level of probability if Σ (λ) is greater than 1 64 $\sqrt{\Sigma (\gamma)}$, and at the 1 in 100 level if Σ (λ) is greater than 2 33 $\sqrt{\Sigma (\gamma)}$

In the present material, the Σ (λ) was found to be —5 and the Σ (γ) = 15 (Table 2) This value of Σ (λ) gives no suspicion of any linkage between the Lp and Ag systems

The investigation of a larger number of informative families would be of great interest In view of the small chance to find informative families however the family material would then have to be considerably extended The material investigated till now must be regarded as a preliminary indication that no linkage exists between the Lp and Ag serum type systems

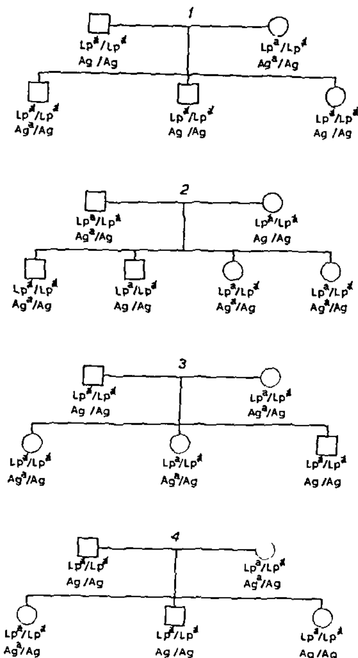


Fig 4

Four "double back cross" families informative for linkage study between the Lp and *Ag* serum type systems of human β lipoprotein Lpª is the designation for the recessive gene within the Lp system and *Ag* for the recessive gene in the Ag system

negative child Consequently, a linkage study can only be made in a material of families where one of the parents is heterozygous for both the genes Lp^a and Ag^a and the other parent lacks both the genes, as the destination of the heterozygous parent's genes in the children is possible only in these families

As a family material already tested for the Lp(a) factor was avail-

and the authors state that the slides should be washed and stained be fore reading The genetic studies performed so far seem incomplete As much as 96 7 per cent of white Americans reacted positively with this new antiserum and the authors found no American family where both the parents were negative Only in a material of Micronesians they were able to find 4 families in which both parents were negative The children of these families were all negative The β lipoprotein factor detected with this new antiserum is called the Ag(b) factor the re lationship between the two genes determining the Ag(a) and Ag(b) fac tors is however not one of simple allelism

So far comparative studies have only been performed with the well investigated Ag(a) factor In the future similar studies should be per formed with regard to the Ag(b) and other β lipoprotein factors which might be detected by means of isoantisera The use of isoantisera does however present difficulties due to their rarity and because of the weakness of the antibody of some of the sera Moreover frequently transfused patients often suffer from conditions prohibiting large blood donations It is therefore reasonable to try to produce heteroantisera against the genetic groups of human serum β lipoprotein

SUMMARY AND CONCLUSIONS

A comparative study of the Lp system as defined by absorbed rabbit immune serum anti Lp(a) serum and the Ag system as defined by anti Ag(a) serum of human serum β lipoprotein is presented

Sera of all possible combinations of Lp and Ag types were found and no antigenic relationship between the Ag(a) and the Lp(a) factors was demonstrable

It was shown that in sera possessing both the Lp(a) and Ag(a) fac tors these two factors referred to different molecules within the β lipo protein

From the experiments described it is obvious that even if the anti Ag(a) serum should contain several different antibodies none of them could be identical to that of the anti Lp(a) serum

The investigation of sera from unrelated individuals gave no sug gestion of any association between the Ag and Lp systems A study of informative families gave no suggestion of linkage between the two systems

It is therefore concluded that the Lp and Ag systems of human serum β lipoprotein are entirely independent and the necessity of a different nomenclature for the Lp and Ag systems is therefore confirmed by the present work

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DISCUSSION

It has earlier been demonstrated that both the Ag(a) and the Lp(a) factors belong to the human serum β-lipoprotein, and that the two factors are genetically determined (*Blumberg, Dray & Robinson* 1962, *Berg* 1963). It was therefore important to investigate whether any interdependency exists between the two systems.

The immunological tests showed that human sera of all combinations of Lp and Ag types exist, and that there is no antigenic relationship between the Lp(a) and Ag(a) factors. The reaction of non-identity between the Lp(a)- and Ag(a)- precipitates, the failing ability of a Lp(a—) Ag(a+) serum to absorb the antibody of an anti-Lp(a)-serum, and the failing ability of a Lp(a+) Ag(a—) serum to absorb the antibody of the anti-Ag(a)-serum are especially significant in this respect.

The experiment designed to study whether the Lp(a) and Ag(a) factors are located on the same β-lipoprotein molecule in persons possessing both factors, seems to prove that such individuals possess one kind of molecules with the Ag(a) but not the Lp(a) factor, and another kind of molecules with the Lp(a) and not the Ag(a) factor.

The testing of sera from unrelated individuals gave no evidence for any association between the Ag(a) and the Lp(a) factors.

The number of families informative for linkage studies between the Lp and Ag systems was small. As far as the available data extend, however, no evidence for linkage between the two systems was found.

It seems worth mentioning, that also a difference in the stability exists between the Lp(a) and Ag(a) factors. Fresh sera should be used for the Lp tests, as the reactions after some 6 months were considerably weaker and may even disappear within one year of storage in the frozen state (see *Berg* 1963). *Allison & Blumberg* (1961), however, found that even 5 years old sera could be used for the tests for the Ag(a) factor.

The observations of the present study provide evidence that the Lp and Ag systems of human serum β lipoprotein are two independent systems concerning one serum protein. The ability of one serum protein to possess factors of two independent genetic systems, has previously been demonstrated for human 7S γ globulin. In this protein, factors belonging to both the Gm and Inv systems are located (*Harboe & Osterland* 1963), and although the Inv factors are also characteristics of other globulins, it seems reasonable to draw a parallel between this phenomenon and the Ag and Lp factors found in β-lipoprotein.

In addition to the anti-Ag(a) serum, *Blumberg & Riddell* (1963) have reported the detection of another serum from a patient who had received multiple transfusions, probably revealing another genetic factor of human serum β-lipoprotein. The reactions obtained with this anti-serum were weaker than those obtained with the anti Ag(a)-serum,

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PRECIPITATION REACTIONS IN AGAR GEL BETWEEN ALBUMIN AND β LIPOPROTEIN OF HUMAN SERUM[1]

By

KÅRE BERG

Received 8 V 64

The agar gel double diffusion technique of Ouchterlony has been widely used in immunological studies (for review, see *Ouchterlony* 1962)

Several authors have stressed the multiplicity of factors that may influence the results of immunodiffusion tests in agar gel (for review, see *Crowle* 1960, 1961)

Precipitates in agar gel may appear, even if no antigen-antibody reaction takes place

Reactions between normal human serum and hemolysed erythrocytes (*Peetoom, Rose, Ruddy, Micheli & Grabar* 1960), and between human albumin and hemoglobin of human tissue extracts (*Metzgar & Grace* 1961), may give precipitates very similar to immune precipitates

Non immune precipitates in agar gel double diffusion tests have also been observed between normal serum and tissue extracts (*Metzgar & Grace* 1961, *Tomasi* 1961, *Berenbaum, Kitch & Cope* 1962) and between serum and extracts of tumours (*Deckers & Maisin* 1963) Recently, *Niece & Barrett* (1963) found that an antigen possessing enzymatic activity may provoke the formation of non immune precipitates in Ouchterlony plates

The purpose of the present paper is to describe another type of non-immune precipitates in agar gel double diffusion experiments, simulating antigen antibody reactions

It is known that β lipoprotein may interact with the agar medium, and a special type of agar, K agar, has been used for a quantitative precipitation test for human serum β lipoprotein (*Boyle & Moore* 1959) With ordinary laboratory grade agar, a circular precipitate may arise around the well containing serum β lipoprotein, especially if the agar is dissolved in barbital buffer (*Crowle* 1961) The utmost care is therefore necessary in performing agar gel diffusion tests with isolated β-lipoprotein

During the work on a new serum type system concerning human

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[1] Aided by a grant from *Dr A Wilthes Legat*

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It is known that β lipoprotein may interact with the agar medium, and a special type of agar, K agar has been used for a quantitative precipitation test for human serum β lipoprotein (*Boyle & Moore* 1959) With ordinary laboratory grade agar a circular precipitate may arise around the well containing serum β lipoprotein especially if the agar is dissolved in barbital buffer (*Crowle* 1961) The utmost care is therefore necessary in performing agar gel diffusion tests with isolated β lipoprotein

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β-lipoprotein (the Lp system, *Berg* 1963), purified β-lipoprotein was tested against different immune sera. In the course of these experiments, the phenomenon to be described was first observed. It was found that isolated β-lipoprotein, when allowed to diffuse against different normal sera, led to the formation of distinct precipitates in the Ouchterlony dishes, thus representing a possibility for misinterpretation of agar gel tests including isolated β-lipoprotein. Further experiments showed that these non-immune precipitates depend upon an interaction between β-lipoprotein and albumin.

MATERIALS AND METHODS

Human Sera

A panel of 10 fresh normal human sera from laboratory personnel was used for the experiments In one experiment, five sera, stored at −20° C for 7 years, were also used

Human β-Lipoprotein.

Fresh, undialysed normal human serum from single individuals was fractionated on a hydroxylapatite column with a stepwise elution technique according to *Hjertén* (1959) Before use, all β-lipoprotein fractions from the chromatographic experiments were dialysed against a 0 85 per cent saline solution The technique is described in detail elsewhere (*Berg* 1963)

To test the reproducibility of the chromatographic method, several batches of β-lipoprotein from two different donors, prepared with the standard chromatographic technique were tested, and all gave the phenomenon to be described The β-lipo protein fractions from 3 chromatographic runs with serum from one person were pooled, concentrated and used for the experiments to be described

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dry [illegible]
and [illegible]
volume of dialysate This β-lipoprotein solution did not contain other serum proteins detectable by immunoelectrophoresis

In addition to the β-lipoprotein prepared by chromatography, a sample of this lipoprotein prepared in the ultracentrifuge was used This was Op -Nr 22263 from Behringwerke AG, Marburg[1]

Solution of all Human Serum Proteins Except β-Lipoprotein

For the production of this solution, the two step fractionation technique of *Cramer* (1962) on the hydroxylapatite column was applied By means of this technique all serum proteins except the β-lipoprotein were eluted from 8 5 ml of serum by 100 ml 0 25 M sodium phosphate buffer of pH 6 8 This eluate was concentrated to the same volume as that of the serum applied to the column This solution did not react with specific rabbit-anti-human β lipoprotein-antiserum It apparently contained all other serum proteins than β lipoprotein, when tested by immuno electrophoresis

Serum Lacking β Lipoprotein

A sample of serum from a patient with congenital absence of β lipoprotein was kindly supplied by *Professor M Lamy* Paris This is patient number 1 in the publication of *Lamy Frézal Polonovski Druez* & *Rey* (1963)

Human Albumin

Crystalline human albumin ("Reinste" Behringwerke AG Marburg Op -Nr 1916) was dissolved in a 0 85 per cent saline solution to obtain the following concen

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1 I am indebted to *Dr Heide* and *Dr Störiko* Behringwerke AG, Marburg for providing this sample

trations (w/v) of albumin 4 per cent 2 per cent, 1 per cent, 0 1 per cent 0 01 per cent When nothing else is particularly stated the 1 per cent solution was used The albumin solutions did not contain other serum proteins detectable by immuno electrophoresis

Human γ Globulin

A one per cent solution of human γ globulin prepared by chromatography on DEAE cellulose was kindly provided by *Dr T Reinskou* Institute of Forensic Me dicine University of Oslo The γ globulin solution did not contain other serum proteins detectable by immunoelectrophoresis

Bovine Albumin

Bovine albumin (30 per cent solution Armour Pharmaceutical Company Ltd Eastbourne England) was diluted with 0 85 per cent saline to 6 per cent 3 per cent, 1 per cent and 0 1 per cent solutions

Normal Rabbit Serum

Serum from a healthy not immunized 3 months old albino rabbit was used

Rabbit Anti Human Serum Antiserum

Anti Humanserum (Behringwerke AG Marburg Op Nr 470F) was used as anti serum in the immunoelectrophoretic tests of serum protein fractions

Specific Rabbit Anti Human β Lipoprotein Antisera

The anti β lipoprotein sera were obtained by immunization of rabbits with iso lated β lipoprotein using the immunization technique described by *Berg* (1963) When these sera were absorbed with serum from a patient with congenital absence of β lipoprotein (one part of the patient s serum to 20 parts of rabbit immune se rum) they reacted only with the β lipoprotein and no other proteins of human serum

Specific Anti Lp(a) Antiserum from Rabbit

Serum from rabbit K 3 absorbed in the proportion 2 5 with human serum of type Lp(a—) (*Berg* 1963) was used

Dialysis Bags

Cellophane casings from Visking Co Chicago Ill were used

Concentration of Chromatographic Fractions

Eluted fractions from chromatographic columns were concentrated by dialysis against Polyethyleneglycol 20000 (Hoechst) as described by Kohn (1959)

Petri Dishes

ANUMBRA dishes 7 cm in diameter were used for agar gel double diffusion tests

Agar

Difco Bacto Agar (Control No 452925) was used (Difco Laboratories Detroit 1, Mich)

Agar Gel Double Diffusion Tests

Each Petri dish was filled with 24 ml 1 per cent (w/v) agar containing 0 85 per cent (w/v) NaCl 10 per cent (v/v) 1/15 M sodium phosphate buffer pH 7 0 and 1 10000 (w/v) merthiolate This was the standard mixture for the agar dishes in the experiments to be described The effect of the different reagents of the gel was further tested as described under Experiments and Results

In the agar gel 6 peripheral wells were cut around one central well and the bottom of each well was sealed with a thin layer of agar The diameter of each well was 4 mm and the distances between the circumferences of neighbouring wells were in all cases 5 mm The reagents to be tested were distributed in the wells The dishes were kept in a moist chamber at 37° C in the standard procedure Readings of precipitates were made against a dark background by oblique illumination from below after 2, 3 4 5 6 10, 12 14 24 36 and 48 hours and after 3 and 7 days All double diffusion experiments were made in duplicate Some of the dishes were stained with Oil Red O as described by *Uriel* (1960)

Some dishes were stained for esterase activity, as described by *Uriel* (1961)

Immunoelectrophoresis

Immunoelectrophoretic control of the purity of serum protein fractions were performed according to *Grabar & Williams* (1953) with the micromodification described by *Scheidegger* (1955)

Photographic Registrations

Photographic registrations of gel diffusion and immunoelectrophoretic tests were made with a Leica 35 mm camera on Agepe Agfa film

EXPERIMENTS AND RESULTS

Occurrence of Precipitate between Isolated β-Lipoprotein and Whole Human Serum

When two neighbouring wells of a standard agar dish were filled with 60 µl of whole human serum and 0 9 per cent β-lipoprotein solution respectively and the dish was incubated at 37° C, a precipitate became visible after approximately 3 hours The precipitate was located about ⅓ way from the β-lipoprotein well to the well containing human serum The precipitate was at first very sharp and dense, completely simulating a real immune precipitate (See Fig 1) Some 5 hours after the start of the experiment, the precipitate became broader, less dense, and seemed to move somewhat in the direction of the β-lipoprotein well The precipitate became steadily looser, and disappeared completely 10–12 hours after the start of the experiment

These precipitates appeared against all the ten fresh human sera tested and also against all of 5 sera that had been stored for 7 years

The change in density, and the subsequent disappearance of the precipitates, distinguished them clearly from true immune reactions, as for instance the reactions between human sera and a specific anti-Lp(a)-antiserum or anti-β-lipoprotein-antiserum

Identification of the Factor in Normal Whole Serum Reacting with Isolated β-Lipoprotein

As the precipitates only occurred between wells containing isolated β-lipoprotein and albumin or serum, and were not of a circular shape, it was concluded that they were not produced only through an interaction between β-lipoprotein and agar

When a 1 per cent solution of human albumin was placed in the well next to the β-lipoprotein, it was found that this reagent produced a pre-

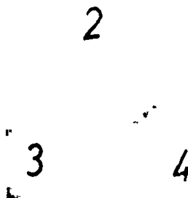


Fig 1

Precipitation in agar gel double diffusion test between isolated β lipoprotein and albumin of human serum

The reagents were

1 Saline solution 0 85 per cent
2 Whole human serum
3 Human albumin 1 per cent solution
4 Human β lipoprotein 0 9 per cent solution

Note reaction of identity between the two precipitates near the well containing human β lipoprotein

(The picture shows only the essential part of a standard agar dish)

cipitate against the 0 9 per cent β lipoprotein solution This precipitate behaved in exactly the same manner as those obtained with whole human serum When isolated β lipoprotein was tested simultaneously against whole human serum and isolated human albumin, a reaction of identity was formed between the two precipitates (Fig 1)

Isolated β lipoprotein was also tested against a 0 85 per cent saline solution, against another sample of isolated β lipoprotein from the same batch and against a 1 per cent solution of γ-globulin No precipitate developed in any of these control experiments Neither did any precipitate occur between pooled isolated albumin and the whole human serum used for isolation of the β lipoprotein, nor when this and other normal human sera were tested against each other

To test if the precipitates could be produced with albumin from other species the β lipoprotein was also tested against bovine albumin and whole normal rabbit serum A precipitate was obtained with both these reagents

It was concluded that the factor in human serum precipitating β lipoprotein was albumin It was further concluded that the β lipoprotein had to be isolated, at least from the albumin, for the occurrence of the precipitate while the albumin took part in the reaction, whether in the isolated state or in whole human serum It was also concluded that not

In the agar gel 6 peripheral wells were cut around one central well and the bottom of each well was sealed with a thin layer of agar The diameter of each well was 4 mm and the distances between the circumferences of neighbouring wells were in all cases 5 mm The reagents to be tested were distributed in the wells The dishes were kept in a moist chamber at 37° C, in the standard procedure Readings of pre cipitates were made against a dark background by oblique illumination from below after 2, 3, 4 5 6, 10, 12 14, 24, 36 and 48 hours and after 3 and 7 days All double diffusion experiments were made in duplicate Some of the dishes were stained with Oil Red O as described by *Uriel* (1960)

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When a 1 per cent solution of human albumin was placed in the well next to the β-lipoprotein, it was found that this reagent produced a pre-

From the experiments with β lipoprotein and albumin in differe concentrations it was found that while the 1 per cent albumin g very distinct precipitates, the 0 1 per cent solution gave only a trace a precipitate occurring after 24 hours, and the 0 01 per cent solut gave no precipitate at all

When the standard β-lipoprotein solution was diluted 1/8 it still g a distinct precipitate, while the 1/16 dilution only gave a trace of a p cipitate, visible after 24 hours

Some of the dishes containing long lasting precipitates were was for a few hours, dried, and stained with Oil Red O The precipitates not disappear during washing, and were stained red with this reag demonstrating the presence of lipid in the precipitates

When stained for esterase activity, the precipitates became red, dicating the presence of such activity

In none of the experiments described were any precipitates vis after 36 hours or later When the precipitates once had disappea they never returned

"Absorption Experiment

An experiment to investigate if the precipitating ability could be moved from the albumin solution through "absorption" with β l protein was made One well was filled with the 0 9 per cent β- protein solution, and the dish was placed in a moist chamber until solution had diffused into the surrounding agar gel The well was filled with 1 per cent albumin solution and a neighbouring well 0 9 per cent β lipoprotein solution No precipitate developed after albumin solution had been submitted to this 'intra basin absorpt with β lipoprotein before diffusing against the β lipoprotein of neighbouring well The phenomenon studied in this work thus beh like true immune precipitation, also with respect to the effe 'absorption'

Effect of Salt Concentration in the Agar Gel on Albumin β-Lipopr Precipitate Formation

To test the effect of the salt concentration of the agar gel, an e iment was performed, in which the concentration of NaCl in the gel was varied, all other conditions being kept constant The diff concentrations of NaCl are listed in Table 3

The diffusing reagents were 1 per cent albumin solut[illegible] and 0

the ge

' lipop

[illegible]concentration of 2 per cent or higher did not pr the development of true immune precipitates

only human albumin, but also bovine and probably also rabbit albumin, could precipitate human β-lipoprotein

Quantitative Studies of the Interaction between Albumin and β-Lipoprotein

To test the effect of the amount of the two reagents on the development of precipitate, different dilutions of the 0 9 per cent β-lipoprotein solution were tested against a 1 per cent solution of human albumin, and the 0 9 per cent solution of β-lipoprotein was tested against different concentrations of human and bovine albumin The results are shown in Tables 1 and 2

TABLE 1

Reaction Pattern by Double Diffusion in Agar Gel between Human Albumin and β-Lipoprotein

Human serum Albumin	Dilutions of a 0 9 per cent β lipoprotein solution					
	1 1	1 2	1 4	1 8	1 16	1 32
1 per cent solution	+	+	+	(+)	±	—

+ = distinct precipitate
(+) = weak precipitate
± = very weak (and late occurring) precipitate
— = no visible precipitate

TABLE 2

Reaction Pattern by Double Diffusion in Agar Gel between Human β Lipoprotein and Albumin

Human β lipoprotein solution	Concentrations of solutions of albumin								
	Human					*Bovine*			
	4 %	2 %	1 %	0 1 %	0 01 %	6 %	3 %	1 %	0 1 %
0 9 per cent	+	+	+	±		+	+	+	—

For significance of + ± and — see Table 1

It was found that while 1 per cent albumin gave precipitates behaving very like those obtained with whole human serum, the stronger albumin solutions had a tendency to give more long-lasting precipitates This was particularly obvious when the 4 per cent and 2 per cent human albumin and the 6 per cent bovine albumin solutions were used With these reagents the precipitates were visible even after 24 hours Whole serum from a patient with congenital absence of β-lipoprotein and the fraction from the hydroxylapatite chromatography of human serum eluted with 0 25 M phosphate buffer with a pH of 6 8 behaved very similar to the stronger albumin solutions, with more long-lasting precipitates

From the experiments with β lipoprotein and albumin in different concentrations it was found that while the 1 per cent albumin gave very distinct precipitates the 0 1 per cent solution gave only a trace of a precipitate occurring after 24 hours and the 0 01 per cent solution gave no precipitate at all

When the standard β lipoprotein solution was diluted 1/8 it still gave a distinct precipitate while the 1/16 dilution only gave a trace of a precipitate visible after 24 hours

Some of the dishes containing long lasting precipitates were washed for a few hours dried and stained with Oil Red O The precipitates did not disappear during washing and were stained red with this reagent demonstrating the presence of lipid in the precipitates

When stained for esterase activity the precipitates became red indicating the presence of such activity

In none of the experiments described were any precipitates visible after 36 hours or later When the precipitates once had disappeared they never returned

Absorption Experiment

An experiment to investigate if the precipitating ability could be removed from the albumin solution through absorption with β lipoprotein was made One well was filled with the 0 9 *per cent* β *lipoprotein* solution and the dish was placed in a moist chamber until this solution had diffused into the surrounding agar gel The well was then filled with 1 per cent albumin solution and a neighbouring well with 0 9 per cent β lipoprotein solution No precipitate developed after the albumin solution had been submitted to this intra basin absorption with β lipoprotein before diffusing against the β *lipoprotein of the* neighbouring well The phenomenon studied in this work thus behaved like true immune precipitation also with respect to the effect of absorption

Effect of Salt Concentration in the Agar Gel on Albumin β Lipoprotein Precipitate Formation

To test the effect of the salt concentration of the agar gel an experiment was performed in which the concentration of NaCl in the agar gel was varied all other conditions being kept constant The different concentrations of NaCl are listed in Table 3

The diffusing reagents were 1 per cent albumin s[illegible] and 0 9 per [illegible] the gel was [illegible] lipoprotein [illegible] ncentration of 2 per cent *or higher* did not prevent the development of true immune precipitates

[illegible]

only human albumin, but also bovine and probably also rabbit albumin, could precipitate human β-lipoprotein

Quantitative Studies of the Interaction between Albumin and β-Lipoprotein

To test the effect of the amount of the two reagents on the development of precipitate, different dilutions of the 0.9 per cent β-lipoprotein solution were tested against a 1 per cent solution of human albumin, and the 0.9 per cent solution of β-lipoprotein was tested against different concentrations of human and bovine albumin. The results are shown in Tables 1 and 2.

TABLE 1

Reaction Pattern by Double Diffusion in Agar Gel between Human Albumin and β-Lipoprotein

Human serum Albumin	Dilutions of a 0.9 per cent β-lipoprotein solution					
	1:1	1:2	1:4	1:8	1:16	1:32
1 per cent solution	+	+	+	(+)	±	—

+ = distinct precipitate
(+) = weak precipitate
± = very weak (and late occurring) precipitate
— = no visible precipitate

TABLE 2

Reaction Pattern by Double Diffusion in Agar Gel between Human β-Lipoprotein and Albumin

Human β-lipoprotein solution	Concentrations of solutions of albumin								
	Human					Bovine			
	4%	2%	1%	0.1%	0.01%	6%	3%	1%	0.1%
0.9 per cent	+	+	+	±		+	+	+	—

For significance of + ± and — see Table 1

It was found that while 1 per cent albumin gave precipitates behaving very like those obtained with whole human serum, the stronger albumin solutions had a tendency to give more long lasting precipitates. This was particularly obvious when the 4 per cent and 2 per cent human albumin and the 6 per cent bovine albumin solutions were used. With these reagents the precipitates were visible even after 24 hours. Whole serum from a patient with congenital absence of β-lipoprotein and the fraction from the hydroxylapatite chromatography of human serum eluted with 0.25 M phosphate buffer with a pH of 6.8, behaved very similar to the stronger albumin solutions, with more long-lasting precipitates.

all the other pH values 1/15 M phosphate buffers were used Dishes with the following pH values were prepared 5 0, 6 0, 7 0, 8 0 and 8 6

It was found that the precipitate was most distinct at pH 7 0 but was also recognizable at pH 8 0 and 8 6 although it developed a little later and appeared less dense All these precipitates disappeared after 10–12 hours

At pH 5 0 and 6 0 circular precipitates around the well containing β lipoprotein developed which probably could mask a precipitate be tween albumin and β lipoprotein

Effect of Temperature on the Albumin β Lipoprotein Precipitate Formation

All experiments hitherto described were performed at 37° C To test the effect of temperature, standard agar dishes were kept at 4°, 20° and 37° C The albumin β lipoprotein precipitate developed at all three tem peratures, it was gone after 12 hours at 37° C, at this time still present at 20° C and 4° C, and after 24 hours still distinct at 4° C and also pres ent at 20° C All precipitates had vanished after 36 hours

Experiment with β Lipoprotein Prepared in the Ultracentrifuge

To investigate whether the precipitates described only occurred with β lipoprotein prepared by hydroxylapatite column chromatography, a 0 5 per cent solution of β lipoprotein prepared in the ultracentrifuge was tested against 1 and 2 per cent solutions of human albumin, and against 1 and 6 per cent solutions of bovine albumin A precipitate oc curred between the β lipoprotein and each of the albumin solutions mentioned

DISCUSSION

The experiments described demonstrate that a precipitate in agar gel can occur between albumin and β lipoprotein of normal human serum From this study it seems necessary for the β lipoprotein to be in the isolated state to give the precipitate described whereas the albumin forms precipitate whether isolated or present in whole serum It is at present not known if sera exist in which the β lipoprotein behaves as if it were in the isolated state, or sera which for some reason make β lipoprotein of other sera behave as if it were in the isolated state, thus giving precipitates between whole human sera This author has, for instance, not had the opportunity to study sera from patients with analbuminemia

The precipitate between albumin and β lipoprotein is very like a true immune precipitate, also as to the effect of 'absorption" It is, however, clearly distinguishable from real immune precipitates at least with the composition of the agar gel used as the standard in the present work,

TABLE 3

Reaction Pattern by Double Diffusion in Agar Gel between β Lipoprotein and Albumin of Human Serum at Different Concentrations of NaCl in Agar Gel

Reacting solutions	Concentrations of NaCl in agar gel						
	0 %	0 45 %	0 9 %	2 %	3 %	5 %	10 %
Albumin and β lipoprotein	+	+	+	—	—	—	—

For significance of + and —, see Table 1

This gel contained 1 per cent (w/v) agar, 0 07 M sodium phosphate buffer pH 7 0 and 1/10000 (w/v) merthiolate In agar dishes of this composition, the precipitate between albumin and β-lipoprotein tended to last much longer than in the experiments earlier described in this paragraph The precipitates were distinct even after 24 hours

Effect of the Concentration of Agar in the Gel on the Albumin-β-Lipoprotein Precipitate Formation

Agar dishes with different concentrations of agar, as shown in Table 4, were prepared, all other conditions being kept as described for the standard dish

TABLE 4

Reaction Pattern by Double Diffusion in Agar Gel between β Lipoprotein and Albumin of Human Serum at Different Concentrations of Agar in Gel

Reacting solutions	Concentrations of agar in gel					
	0 5 %	1 %	1 5 %	2 %	3 %	5 %
Albumin and β lipoprotein	+	+	+	+	—	—

For significance of + and — see Table 1

It was found, that when the agar concentration was 3 per cent or more, no precipitate between albumin and β-lipoprotein was formed When the agar concentration was 1 5 per cent, the precipitate developed after 5 hours, and when the concentration was 2 per cent, after about 7 hours When the concentration was 0 5 per cent, the precipitate could still be observed after 24 hours At all other agar concentrations tested, it disappeared 10 12 hours after the experiments were started

Effect of pH on the Albumin-β Lipoprotein Precipitate Formation

In agar dishes of otherwise standard composition, the pH was varied by the addition of buffers of different pH values The volume of the buffer was as given in the standard description The buffer used to obtain a pH of 8 6, was barbital buffer with an ionic strength of 0 1 For

all the other pH values 1/15 M phosphate buffers were used Dishes with the following pH values were prepared 5 0 6 0 7 0, 8 0 and 8 6

It was found that the precipitate was most distinct at pH 7 0 but was also recognizable at pH 8 0 and 8 6 although it developed a little later and appeared less dense All these precipitates disappeared after 10–12 hours

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DISCUSSION

The experiments described demonstrate that a precipitate in agar gel can occur between albumin and β lipoprotein of normal human serum From this study it seems necessary for the β lipoprotein to be in the isolated state to give the precipitate described whereas the albumin forms precipitate whether isolated or present in whole serum It is at present not known if sera exist in which the β lipoprotein behaves as if it were in the isolated state or sera which for some reason make β lipoprotein of other sera behave as if it were in the isolated state thus giving precipitates between whole human sera This author has, for instance not had the opportunity to study sera from patients with analbuminemia

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and when the dishes are kept at 37° C The precipitates are transient, appearing about 3 hours after the start of the experiment, and usually disappearing 7–9 hours later They are thus easily distinguishable from the Lp(a) precipitates (*Berg* 1963), which appear at a time when the albumin-β-lipoprotein precipitates have dissolved The formation of an albumin-β-lipoprotein precipitate is influenced by the composition of the agar gel The most important finding in this connection probably is that a NaCl concentration of 2 per cent or more in the gel completely prevents the formation of the described precipitates, but does not prevent the development of true immune precipitates This gives a practical method to control if a precipitate including human β-lipoprotein represents a true immunological reaction or not

The true nature of the precipitation observed between albumin and β-lipoprotein is, at present, unknown

From the knowledge of the multiple substances, that may precipitate lipoproteins when techniques other than gel diffusion are applied, (*Amenta & Waters* 1960, *Cornwell & Kruger* 1961), a transient complex formation between albumin and β lipoprotein under certain conditions, seems possible The inhibition of precipitate formation when the salt concentration is raised, is consistent with the findings of *Berenbaum, Kitch & Cope* (1962) They concluded that the non-immune precipitates they observed, therefore involved a salt like combination *Tomasi* (1961) also found that his non-immune precipitates were inhibited when the ionic strength was increased, and concluded that electrostatic factors were important for the reaction From the findings in the present work, it is possible that these conclusions of *Tomasi* (1961) and *Berenbaum, Kitch & Cope* (1962) also are valid for the albumin β-lipoprotein precipitation

It is known that albumin has the ability to form non-immune precipitates in agar gel (*Metzgar & Grace* 1961) The present study shows that also β-lipoprotein can take part in the formation of such precipitates It remains to be investigated if any of the earlier reported non immune precipitates are related to the phenomenon described in this paper As to the findings of precipitates between albumin and tissue extracts, it should be mentioned that such extracts may contain considerable amounts of serum proteins, and that β-lipoprotein then, to some extent at least, perhaps could take part in the formation of these non-immune precipitates

The detection of 2 genetic systems within the β-lipoprotein recently (*Allison & Blumberg* 1961, *Berg* 1963) will probably lead to extensive immunological studies of this protein It is necessary to have in mind the phenomenon described in this paper in the study of isolated β-lipoprotein by means of double diffusion in agar gel

The finding of a precipitate in gel diffusion tests does not prove that an antigen-antibody reaction has taken place The possibility that a precipitate is non-immune, should be considered when interpreting the

results of such experiments Experiments to control if a precipitate really represents an antigen antibody reaction should always be performed

SUMMARY AND CONCLUSIONS

1) A precipitate formation in agar gel between β lipoprotein and albumin very like true immune precipitate, is reported
2) So far, the reaction has only been observed when β-lipoprotein is present in the isolated state, whereas albumin can either be isolated or present as a constituent of whole serum
3) In the author's composition of the agar gel, the precipitate is transient, and therefore easily distinguishable from true immune precipitates, when the experiments are performed at 37° C The precipitate may be more long-lasting under other circumstances, and thereby represent a possibility for false interpretation of agar gel double diffusion tests
4) The presence of NaCl in the gel, inhibits the precipitate formation, a concentration of 2 per cent or more prevents it completely
5) The necessity is stressed, not to interpret a precipitation in agar gel as an immunological reaction before the possibility of a non immune precipitate is excluded

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BRIEF REPORT

THE EFFECT OF DIMETHYLCYSTEINE (PENICILLAMINE®) ON THE MOBILITY OF β_{1A} GLOBULIN IN HUMAN SERUM A PRELIMINARY REPORT

By *B Mansa*

β_{1C} globulin is one of the human serum components which is well characterized by immunoelectrophoresis (3) Some relationship of this protein with the complement system is to be expected due to its transformation to β_{1A} globulin which is demonstrable after prolonged storage of sera (6) *Muller Eberhard et al* (6) have described the isolation of the two globulins in question and have characterized them with respect to electrophoretic mobilities and sedimentation coefficients In addition

cillamine®) is presented

Pathological sera were treated with 0 2 M penicillamine at room temperature for two hours at pH 7 0–7 5 prior to immunoelectrophoretic analyses Using polyvalent rabbit antisera against human serum some of the treated sera were found to develop a precipitation line within the intermediate mobility range for γ globulins For further analysis

ficity including

used With thes

unknown compo

β_{1A} globulin and the unknown component were demonstrated as two individual

but after penicillamine treatment it was assumed that there was a correlation with the amount of β_{1A} globulin present in the individual samples Preliminary

Fresh human serum from a healthy

ith hydrazine in a final concentration

buffered saline (pH 7 38) the sample

munoelectrophoretic analysis of the reduced sample demonstrated the presence of the unknown component Furthermore the analyses showed the expected conversion of β_{1C} globulin to β_{1A} globulin as a result of hydrazine treatment In a control experiment in which incubation with saline was substituted for the hydrazine treatment it was impossible to demonstrate the

tr

or

Received 16 vii 64 from the Department of Biophysics Statens Seruminstitut Copenhagen

[1] Commercially available from Centraal Laboratorium van de Bl dienst Het Nederlandsche Roodekruis Ams

β_{1F} globulin are demonstrable by the use o

[2] Rabbit anti FAC1 rabbit C4 human

disposal through the courtesy of prof *P Klein* Mainz

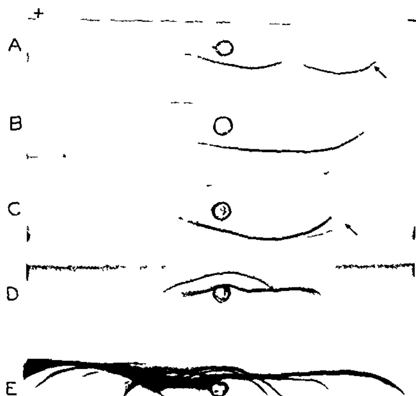


Fig 1 illustrates the immunoelectrophoretic demonstration of the new component γ_{SH} globulin A stored serum sample from a patient with pleuropneumonia which contained β_{1A} globulin was electrophoretically separated after penicillamine treatment (at A B and C) The same untreated serum was separated electrophoretically as a control (at D and E) The following antisera were used A anti C_3C_4 B anti γ_1 macroglobulin C and D a mixture of 3 volumes anti C_3C_4 and 2 volumes anti γ_{1M} globulin E a polyvalent anti human serum The serum sample is seen to contain β_{1A}- and β_{1C} globulin when analysed with the anti C_3C_4 The new component γ_{SH}-globulin was demonstrable within the intermediate γ globulin mobility range after the penicillamine treatment The precipitation line of this component is indicated by arrows in Fig 1 A and C For further details see text

line corresponded to the β_{1E} globulin line developed with the above mentioned anti C_3/C_4 antiserum This result indicates that the unknown component is immunologically related to β_{1A}/C globulin Stored serum samples in which part of the β_{1C} globulin remained unchanged showed after penicillamine treatment a three humped precipitation line the individual parts of which consisted of 1) β_{1A} globulin 2) β_{1C}-globulin and 3) the unknown component The identification of these 3 lines was established by immunoelectrophoretic analysis with the anti C_3/C_4 antiserum in the antibody trough A partial fusion of the lines presented evidence for the immunological relationship between β_{1C} globulin and the unknown component

The chemical nature of the action of penicillamine on β_{1A} globulin is unknown but presumably a reduction of disulphide bonds takes place The change in net charge demonstrated by the decrease in the electrophoretic mobility can probably be explained by assuming that a heavily charged but rather small part of each molecule is split off from the main part and that only the major part of the molecule is demonstrable by the technique used

It is proposed that the unknown component be named γ_{SH} globulin Such a name would give an indication of its mobility range would also show its relationship to

the complement component β_{1C} globulin (C'_{3a}) and finally point out that it appears after treatment with a S S splitting agent

Summary The present study has shown that β_{1A} globulin in human serum is sensitive to the disulphide splitting agent penicillamine This treatment resulted in a considerable decrease in electrophoretic mobility The split product was demonstrable within the intermediate γ globulin mobility range by immunoelectrophoretic analysis Identical results were obtained by treatment of β_{1A} globulin in stored sera and in hydrazine treated sera The new component could not be demonstrated after penicillamine treatment of β_{1C} globulin in fresh serum An immunological relationship to $\beta_{1A/C}$ globulin was established γ_{SH} globulin is proposed as a name for this new component

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BRIEF REPORT

ISOLATION OF THE TRIC AGENT FROM 3 CASES OF NEONATAL INCLUSION BLENNORRHOEA AND FROM THE CERVIX OF ONE OF THE MOTHERS

By *C H Mordhorst*

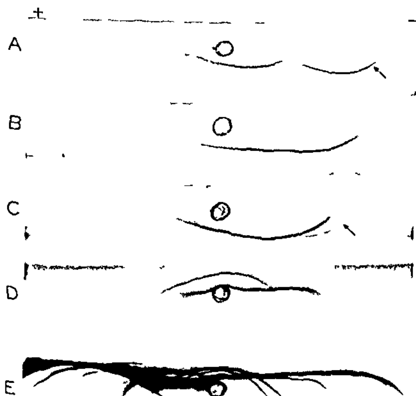


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It is proposed that the unknown component be named γ_{SH} globulin Such a name would give an indication of its mobility range would also show its relationship to

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BRIEF REPORT

IDENTIFICATION OF CANDIDA ALBICANS

By *A Stenderup* and *J Brown Thomsen*

Various methods for the quick identification of candida albicans have been published during recent years In 1960 *Taschdjian et al* published the filamentation test performed in serum and later *Buckley & van Uden* (1963) modified the test using eggwhite instead of serum claiming that eggwhite could be used on slides as well as in tubes They also indicated that the medium on which the yeasts had been cultured might be of significance

Rosenthal & Furnari (1958) used slide agglutination in the identification of *Candida albicans* using specific antiserum, which is now commercially available (Difco)

A "clotting' reaction was described by *Kaminsky & Quinian* in 1963

As we have not been able to o[illegible] different methods we have made a comp[illegible] were used (candida albicans 107, torulopsis [illegible] 196 other

[illegible] used in this laboratory (Brain Heart [illegible]yein and penicillin, Sabouraud agar [illegible] significance for the results obtained

The results obtained when we used the different variations in the filamentation test are given below

Filamentation Test

	Candida albicans (107 strains) positive	negative
Eggwhite (slide technic)	26 (24%)	81 (76%)
Eggwhite (tube technic)	107 (100%)	0
Serum (tube technic)	107 (100%)	0

All other strains (397) showed negative reactions

Results of slide agglutination test are shown below

Candida albicans antiserum

	Reactions (+)	+	++	+++	% positive
C albicans (107)	3	20	70	14	100%
C tropicalis (30)	1	8	3	0	40%
C parapsilosis (38)	0	0	2	0	5%

All other strains showed negative reactions

We have not been able to verify the so called "clotting" reaction, which we found negative in all strains but one

Received 20 viii 64 from the Institute of General Pathology and Bacteriology, University of Aarhus (Heads professor E A Freundt and professor A Stenderup)
Aided by a grant from *Statens almindelige Videnskabsfond*

A typical clinical picture of inclusion blennorrhoea was seen in all five babies examined. Repeated conjunctival scrapings revealed in each case a number of cytoplasmatic inclusion bodies in epithelial cells. Positive isolates were obtained from the conjunctiva of 3 of the 5 babies.

Cervical scrapings were obtained from three of the five mothers with inclusion positive babies. These smears disclosed a cytology very much similar to that of the conjunctiva after infection with the TRIC agent but the presence of inclusion bodies could not be demonstrated. A TRIC isolate was however obtained from one of the three mothers. This woman was the mother of one of the three babies who yielded a positive isolation.

From each positive patient at least 2 isolates were obtained from specimens collected on consecutive days. The strains were usually recognized in the second yolk sac passage and the embryo was killed in this or the third passage. The impression smears of the yolk sac membranes disclosed numerous free elementary bodies. In morphology and colour these elementary bodies were found to resemble those of other members of the psittacosis LGV trachoma group of agents. The egg LD_{50} of the strains after establishing was of the order to $10^{4.5}$ to $10^{6.5}$ per ml. All strains have by now been through 7 to 25 egg passages.

During the same period as the above mentioned isolations were made about 20 young women were examined for a TRIC agent infection of the genital tract. These patients were selected among patients attending a venereal disease clinic because their sera fixed complement to a titer of 1 10 to 1 40 with a LGV antigen. Cervical scrapings from these patients did not indicate a TRIC infection and repeated attempts to isolate an agent from these 20 patients have so far been negative.

Antigen prepared from the isolates by the method described by *Volkert & Møller Christensen* (6) fixed complement with LGV antisera to full titer. Strains from the conjunctiva as well as the strain from the cervix inoculated onto the conjunctival sac of monkeys (*M cynomolgus*) res
intracytoplasmatic inclusion bodies
eyes. The clinical course in the monk
derived from the conjunctiva or from the cervix (7).

One of the strains isolated from the conjunctiva of a child produced by accident a typical picture of inclusion conjunctivitis in a laboratory worker. A number of inclusion bodies was found in conjunctival smears from the affected eye and a TRIC strain was isolated in eggs.

Summary. From 3 out of 5 active cases of neonatal inclusion blennorrhoea in Denmark an agent belonging to the trachoma inclusion conjunctivitis (TRIC) group has been isolated by the chick embryo technic. A similar agent was isolated from the cervix of one of the three mothers.

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Rikshospitalet Oslo Norway (Head Leiv Kreyberg M D)

A STUDY OF EPIDERMAL TUMOURIGENESIS IN THE HAIRLESS MOUSE WITH SINGLE AND WITH REPEATED APPLICATIONS OF 3 METHYLCHOLANTHRENE AT DIFFERENT DOSAGES

By

OLAV HILMAR IVERSEN and ULLA IVERSEN

Received 12 iii 64

A sufficiently large single dose of a carcinogenic hydrocarbon, administered as a surface application, can provoke papillomas, and even carcinomas, in mouse skin (see, for instance, *Findlay* 1925, *Mider & Morton* 1939, *Law* 1941, *Cramer & Stowell* 1943, *Engelbreth-Holm & S Iversen* 1951, *Andreasen & Engelbreth Holm* 1953, *Borum* 1954, *Klein* 1956, *Roe* 1956, *Poel* 1959 and *Terracini, Shubik & Della Porta* 1960)

The last mentioned authors concluded from their experiments that A critical dose level exists at which single application of carcinogens becomes fully effective One of us (*Iversen* 1962) has demonstrated that when single applications of methylcholanthrene at different dosages were used the tetrazolium test for carcinogenicity revealed a "step" between the 1/32 and 1/16 per cent solutions The test was negative with solutions of 1/32 per cent and lower, whereas it was positive at concentrations of 1 16 per cent and higher

The present study was undertaken (i) to see if the critical dose level demonstrated for the action of DMBA on Swiss mice by *Terracini, Shubik & Della Porta* could also be observed for methylcholanthrene on hairless mice (ii) to see if a possible critical dose level, as measured by the tumour yield, might be observed at the same dose level as the step in the tetrazolium test, and (iii) to observe if there is some characteristic difference between the results when single and when repeated doses are administered

MATERIALS AND METHODS

A total of 330 hairless mice of the strain hr hr were used The animals were housed in wooden cages and fed our standard diet The —

From this it must be concluded that the filamentation test is a quick and reliable test in the identification of candida albicans when the reaction is performed in tubes using either serum or eggwhite The slide technic with eggwhite is unreliable The slide agglutination test is (in agreement with *Rosenthal & Furnari*) less reliable due to cross reactions especially with strains of *Candida tropicalis*

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A STUDY OF EPIDERMAL TUMOURIGENESIS IN THE HAIRLESS MOUSE WITH SINGLE AND WITH REPEATED APPLICATIONS OF 3-METHYLCHOLANTHRENE AT DIFFERENT DOSAGES

By

OLAV HILMAR IVERSEN and ULLA IVERSEN

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A sufficiently large single dose of a carcinogenic hydrocarbon, administered as a surface application, can provoke papillomas, and even carcinomas, in mouse skin (see, for instance, *Findlay* 1925, *Mider & Morton* 1939, *Law* 1941, *Cramer & Stowell* 1943, *Engelbreth Holm & Iversen* 1951, *Andreasen & Engelbreth Holm* 1953, *Borum* 1954, *Klein* 1956 *Roe* 1956, *Poel* 1959 and *Terracini, Shubik & Della Porta* 1960) The last mentioned authors concluded from their experiments that 'A critical dose level exists at which single application of carcinogens becomes fully effective' One of us (*Iversen* 1962) has demonstrated that when single applications of methylcholanthrene at different dosages were used the tetrazolium test for carcinogenicity revealed a "step" between the 1/32 and 1/16 per cent solutions The test was negative with solutions of 1/32 per cent and lower, whereas it was positive at concentrations of 1/16 per cent and higher

The present study was undertaken (i) to see if the critical dose level demonstrated for the action of DMBA on Swiss mice by *Terracini, Shubik & Della Porta* could also be observed for methylcholanthrene on hairless mice (ii) to see if a possible critical dose level, as measured by the tumour yield might be observed at the same dose level as the "step" in the tetrazolium test and (iii) to observe if there is some characteristic difference between the results when single and when repeated doses are administered

MATERIALS AND METHODS

A total of 330 hairless mice of the strain hr hr were used The animals were housed in wooden cages and fed our standard [illegible]

From this it must be concluded that the filamentation test is a quick and reliable test in the identification of candida albicans when the reaction is performed in tubes using either serum or eggwhite. The slide technic with eggwhite is unreliable.

The slide agglutination test is (in agreement with *Rosenthal & Furnari*) less reliable due to cross reactions, especially with strains of *Candida tropicalis*.

References: *Buckley Helen R. & van Uden N.*: Sabouraudia 2, 205, 1963.—*Kaminsky D. & Quinlan J.*: American Journal of Clinical Pathology 40, 143, 1963.—*Rosenthal S. A. & Domenica Furnari*: Journal of Investigative Dermatology 31, 251, 1958.—*Taschdjian Claire L., Burchall M. A. & Kozinn Philip J.*: A.M.A. Journal of Diseases of Children, 99, 212, 1960.

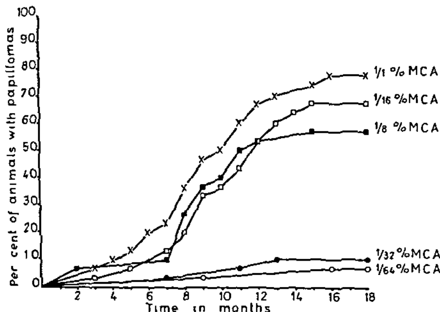


Fig 1

Percentage of tumourbearing animals during the observation period after one single painting with methylcholanthrene in benzene at different concentrations

b) *Single application of methylcholanthrene* The results are presented in Table 2 and in Fig 1 It is seen that whereas the two lowest dosages *viz* the 1/64 and the 1/32 per cent provoked very few papillomas in a low percentage of the mice the three higher dosages produced many papillomas in many of the animals Evidently, the dose/response curve shows a pronounced increase between the two dosages 1 32 and 1/16 per cent corresponding to a borderline application level of roughly 50 micrograms

There is strikingly little difference between the effects of 63, 125 and 1000 micrograms of methylcholanthrene These curves in Fig 1 seem to have a sigmoid shape

The mean latency time which in this work is calculated as the mean time between the treatment and the occurrence of each single tumour, varies very little between the three highest dosages but is a little longer for the few papillomas in the groups with the two lower dosages The average time of occurrence of the first three papillomas however, is much longer for the two lowest dosages than for the three higher doses The two highest dosages provoked a small number of carcinomas

c) *Five consecutive applications of methylcholanthrene* The results are presented in Table 3 and in Fig 2 It is seen that only the lowest concentration 1 64 per cent, provoked relatively few papillomas in few of the mice The five applications of 1 32 per cent solution resulted in a

dilutions from the first solution shortly before the applications An amount of 0.1 ml was applied on the dorsum of each animal by a pipette The solution spread evenly out on the skin after application Table 1 shows the amount of methyl cholanthrene administered at the different experiments Repeated applications were given with three days intervals

TABLE 1

Amount of 3-methylcholanthrene Administered to each Animal at the Different Experiments

Per cent solution	Single application µg	Five applications µg
1/1	1000	—
1/2	—	2500
1/8	125	625
1/16	63	315
1/32	32	160
1/64	16	80

For each type of experiment a group of 30 animals was used 15 males and 15 females (No significant differences between the responses of the two sexes were observed All results are therefore given for both sexes in common)

The animals were examined every 10th day during the observation period of 18 months (78 weeks) Each tumour was recorded and registered as tumour when present for more than 10 days The animals were kept until death or killed after 18 months observation Whenever possible (*i e* except for advanced autolysis) an autopsy was performed and the tumours were controlled histologically

To secure the non carcinogenicity of the solvent a control group of 30 mice was given 20 applications of benzene with ten days intervals and observed as described above

As conclusions are only drawn from differences that are quite evident, no formal statistical calculations of significance are performed

RESULTS

a) *Benzene applications* No papillomas appeared in this group One of the animals developed an ulcer on the dorsum after 10 months This was the only gross evidence of a possible toxicity of benzene to the skin The ulcer healed up after some weeks, and the animal lived for 2 months more without developing any tumour

TABLE 2

Papillomas Appearing on Hairless Mice Skin up to 18 Months after a Single Application of Different Doses of 3 metylcholanthrene in Benzene Solution

Methylcholanthrene concentration in per cent	Total number of tumours per number of mice alive at appearance of first tumour	Per cent of mice bearing		Average time of occurrence of three first papillomas (in months)	Mean latency time (in months)
		Papillomas	Carcinomas		
1/64	3/29	7	0	12.5	12.5
1/32	5/29	10	0	10.0	10.2
1/16	55/30	67	0	5.0	9.4
1/8	43/30	57	3	3.7	8.4
1/1	79/30	77	3	3.5	8.7

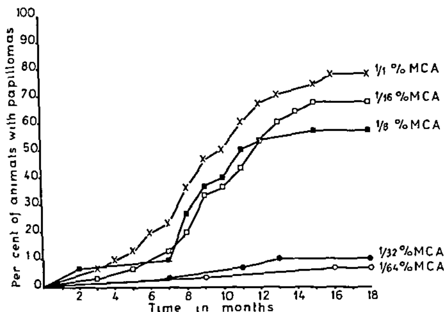


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c) *Five consecutive applications of methylcholanthrene* The results are presented in Table 3 and in Fig 2 It is seen that only the lowest concentration 1 64 per cent provoked relatively few papillomas in few of the mice The five applications of 1 32 per cent solution resulted in a

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Per cent solution	Single application µg	Five applications µg
1/1	1000	—
1/2	—	2500
1/8	125	625
1/16	63	315
1/32	32	160
1/64	16	80

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Methylcholanthrene concentration in per cent	Total number of tumours per number of mice alive at appearance of first tumour	Per cent of mice bearing Papillomas	Per cent of mice bearing Carcinomas	Average time of occurrence of three first papillomas (in months)	Mean latency time (in months)
1/64	3 29	7	0	12 5	12 5
1/32	5 29	10	0	10 0	10 2
1/16	55 30	67	0	5 0	9 4
1/8	43/30	57	3	3 7	8 4
1/1	79/30	77	3	3 5	8 7

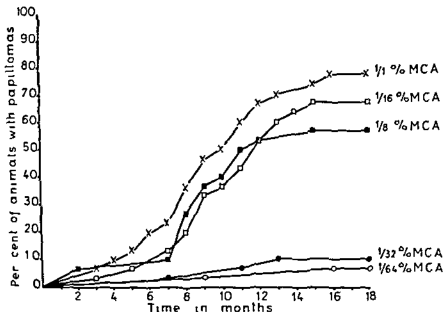


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Percentage of tumourbearing animals during the observation period after one single painting with methylcholanthrene in benzene at different concentrations

b) *Single application of methylcholanthrene* The results are presented in Table 2 and in Fig 1 It is seen that whereas the two lowest dosages, *viz* the 1/64 and the 1/32 per cent, provoked very few papillomas in a low percentage of the mice, the three higher dosages produced many papillomas in many of the animals Evidently, the dose/response curve shows a pronounced increase between the two dosages 1 32 and 1/16 per cent corresponding to a borderline application level of roughly 50 micrograms

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dilutions from the first solution shortly before the applications. An amount of 0.1 ml was applied on the dorsum of each animal by a pipette. The solution spread evenly out on the skin after application. Table 1 shows the amount of methylcholanthrene administered at the different experiments. Repeated applications were given with three days intervals.

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1/64	16	80

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TABLE 2

Papillomas Appearing on Hairless Mice Skin up to 18 Months after a Single Application of Different Doses of 3-metylcholanthrene in Benzene Solution

Methylcholanthrene concentration in per cent	Total number of tumours per number of mice alive at appearance of first tumour	Per cent of mice bearing Papillomas	Per cent of mice bearing Carcinomas	Average time of occurrence of three first papillomas (in months)	Mean latency time (in months)
1/64	3/29	7	0	12.5	12.5
1/32	5/29	10	0	10.0	10.2
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1/1	79/30	77	3	3.5	8.7

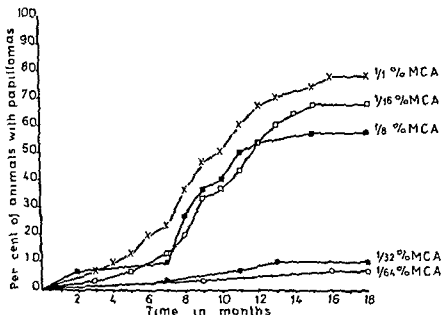


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Percentage of tum urbearing animals during the observati n period after one single painting with methylcholanthrene in benzene at different concentrations

b) *Single application of methylcholanthrene* The results are presented in Table 2 and in Fig 1 It is seen that whereas the two lowest dosages *viz* the 1 64 and the 1 32 per cent provoked very few papillomas in a low percentage of the mice, the three higher dosages produced many papillomas in many of the animals Evidently, the dose/response curve shows a pronounced increase between the two dosages 1 32 and 1/16 per cent corresponding to a borderline application level of roughly 50 micrograms

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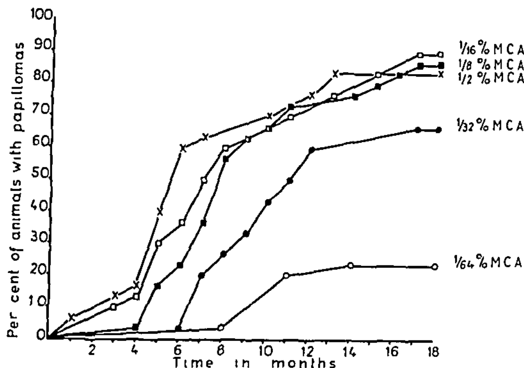


Fig 2

Percentage of tumourbearing animals during the observation period after five repeated paintings with methylcholanthrene in benzene at different concentrations

TABLE 3

Papillomas Appearing on Hairless Mice Skin up to 18 Months after Five Consecutive Applications of Different Doses of 3 methylcholanthrene in Benzene Solution Three Days Intervals between the Applications

Methylcholanthrene concentration in per cent	Total number of tumours per number of mice alive at appearance of first tumour	Per cent of mice bearing — Papillomas	Per cent of mice bearing — Carcinomas	Average time of occurrence of three first papillomas (in months)	Mean latency time (in months)
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1/32	41/21	67	0	6 0	8 0
1/16	90/30	90	3	3 5	8 0
1/8	111/25	88	8	4 7	8 7
1/2	140/17	82	30	3 0	6 3

curve (Fig 2) very similar to that obtained after a single application of 1/16 or of 1 8 per cent (Fig 1)

The latency time is a little longer for the lowest dosage than for the others There is however, no significant differences between the latency time after a single and after five applications The average time of occurrence of the three first tumours is also here shorter for the higher dose levels, but there is no marked step between any of the dosages There is remarkably little difference between the average times of occurrence of the three first papillomas after a single and after five

applications The three highest dosages provoked some carcinomas the yield coming up to 30 per cent after ½ per cent solution

In this group with five applications the mortality of the animals is generally somewhat increased

DISCUSSION

When a carcinogenic hydrocarbon dissolved in benzene is applied to the surface of mouse skin a certain amount of it penetrates into the epidermis and some of it further into the corium where a part is resorbed into the general circulation and finally excreted Many authors have demonstrated that *carcinogens remain in the skin for some time after application* This carcinogen is eliminated partly through metabolism in the epidermal cells themselves partly through continuous diffusion into the corium and partly through the loss of carcinogen loaded cells from the surface (a more detailed discussion of the fate of the carcinogens after topical application is given by *Norden* 1953 and by *Iversen & Evensen* 1962) An average estimate of the presence of carcinogens in the epidermis after a single application is 7 to 9 days (*Engelbreth Holm & S Iversen* 1951) *Poel* (1959) studied the fluorescence after a single drop of carcinogen it remained for 1 to 3 weeks

Probably there is a positive correlation between the dose applied and the time of persistence in the skin *Norden* (1953) concluded after his experiments with 3 4 benzpyrene that the length of time during which benzpyrene or its metabolite is present in the epithelial cells is correlated to the amount of benzpyrene applied and to the amount of lipids in the depots (surface hair canals and sebaceous glands)

Also other factors seem to influence the time of persistence of carcinogens in the epidermis In the haired mice the hair cycle is of great importance It has been shown by *Berenblum Haran Ghera & Trainin* (1958) that the carcinogens remain much longer in the skin when applied during the resting phase than during the growth phase This gives a possible explanation of the "hair cycle effect extensively discussed by Engelbreth Holm and his school (see *Borum* 1960) A moderate single dose of a carcinogen gives rise to many tumours when applied during the resting phase whereas very few or no tumours are seen when the carcinogen is applied during the growth phase With very small doses however no difference in the results can be observed and with massive doses the difference is also masked *Andreasen & Borum* (1959) have demonstrated that also the grade of the early morphological signs of cellular destruction which follows a single application of a moderate dose of DMBA is dependent on the phase of the hair cycle at the time of application This may be interpreted in the same way i e that also the early cell destruction is dependent upon the time of persistence of the carcinogens in the epidermis

As regards the hairless mice used in our experiments the hair cycle effect is probably of no or minor importance The single exposures used

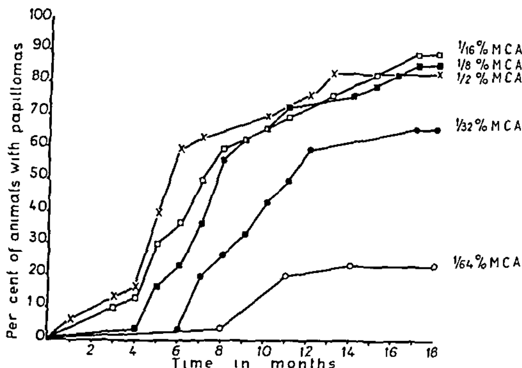


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curve (Fig 2) very similar to that obtained after a single application of *1/16 or of 1 8 per cent* (Fig 1)

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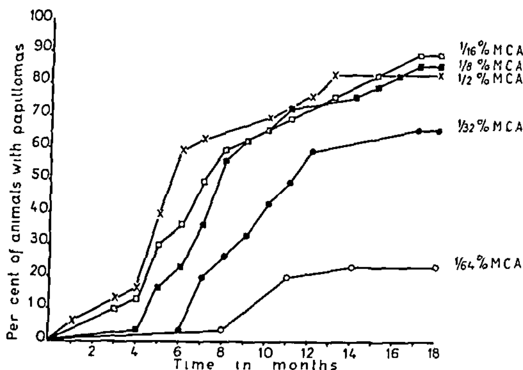


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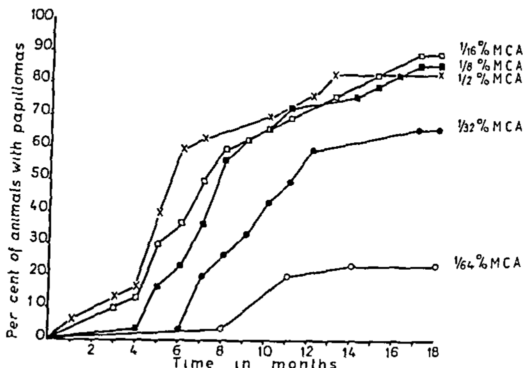


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exposure to carcinogens which takes the hair cycle into consideration, which uses enough animals in each experimental group, and which uses a long enough observation time

The second problem we set out to study was if the possible critical dose level as regards the tumour yield was located at the same dose niveau as the "step" in the tetrazolium test (ii) This was found to be the fact The critical dose level is in both investigations located between the concentrations of 1/32 and 1 16 per cent methylcholanthrene in benzene *Evensen* (1962) has also obseerved a similar "step" in the effect of a single application of different doses of methylcholanthrene on the rate of cell proliferation in the epidermis *Iversen & Evensen* (1962) have interpreted the changes they observe as being signs of cellular destruction, followed by regenerative processes (see also *Iversen & Bjerknes* 1963)

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in our experiments really meant that the epidermal cells were under the continuous, though probably decreasing, influence of carcinogens for 1 to 3 weeks In the experiments with 5 applications with 3 days intervals the exposure was at least two weeks longer

It is also evident that after a single surface application of a certain amount of a carcinogen, we do not know the exact effective dose, *i e* the number of carcinogenic molecules that reaches the supposed critical sites in the cells At least with higher doses an unknown amount of carcinogen is left on the surface and seems not to penetrate the cells It may be assumed that the mean part of this surface-carcinogen is eliminated from the animal by the acute cell loss demonstrated by *Iversen & Evensen* (1962) and later by *Skjæggestad* (1964) But it may probably be permissible to assume that the real effective dose is positively correlated to the applied dose up to a certain dose level Further increase in the dose applied is, however, probably not followed by a proportional rise in the effective dose Only one group of workers (*McCarter, Szerb & Thompson* 1956) has tried to overcome this by using a method which is supposed to give a well-defined dose, washing off the superfluous of carcinogen after a controlled time of application But these authors have not tested the effect of a single dose On the contrary, after using a lot of work to apply an initiating controlled dose of a strong carcinogen, they proceeded by applying repeated, uncontrolled doses of an illdefined, weak carcinogen, the croton oil, thereby destroying any possibility to observe the effects of a supposed short exposure to a carcinogen at controlled dose levels (See also *Ball & McCarter* 1960)

As regards the lower dose levels used in our experiments, we may assume a proportionality between the applied dose and the effective dose For the higher dose levels this proportionality is probably lacking This seems to be the most reasonable explanation of the fact that there is very little difference between the effects of the three highest doses given in our experiments

With these reservations and this definition of the meaning of a single application, we may conclude that the present study (i) confirms the findings of *Terracini, Shubik & Della Porta* (1960) of the critical dose level at which the carcinogens become fully effective after a single application Such a critical dose level is in our experiments observed for methylcholanthrene in benzene solution applied on hairless mice The critical dose level with the technique used is found to be around 50 μg It is manifested as a substantial increase in the total numbers of papillomas occurring in the percentage of animals with tumours and in a possible difference in the average time of occurrence of the three first papillomas in each animal group One may ask why earlier investigations have not revealed this fact A critical examination of the reports, however, makes it clear that the work of *Terracini, Shubik & Della Porta* is the first study of the dose-response curve after a single

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in 67 per cent of the animals This difference may depend on the longer persistence of the carcinogen after five applications But it may as well depend on the higher effective dose level that might be reached after five applications It would obviously have been of interest to study the effects of a few repeated applications of small doses delivered with much longer intervals In that way the probable accumulation of carcinogen with subsequent increase in the effective dose level would be evided, and a relatively stable, long-time effect of a small effective dose could be attained

A possible, simple, but of course more or less speculative explanation of the critical dose level may be the following To provoke the changes in the skin that lead to tumour formation, it is necessary that a certain amount of carcinogenic molecules attack a certain amount of critical sites in the cells These cells are supposed to be the basal cells of the epidermis and its derivatives, or some cells in the corium To reach these cells the carcinogen must diffuse through the horny layer and through the differentiating cells When a certain dose level is applied, enough carcinogen reaches the critical sites and persists in the skin for a sufficiently long time Further increase in the concentration of the solution applied has only slight additional effect because the effective dose is not proportional to the applied dose at higher dose levels With even further increase in the concentration of carcinogen, the direct toxic effect will dominate, and ulcers develop It is well known that papillomas and carcinomas usually develop in the rand zone of such ulcers, where the dose might have been sufficiently high, but non-ulcerative

This way of reasoning refers only to a single painting The results of the five paintings, as well as the massive evidence from the literature, clearly demonstrate that by repeating the applications it is with the strong carcinogens possible to increase the tumour yield up to nearly 100 per cent

Such a simple explanation of the critical dose level after a single application may get some support also by the results obtained after five applications It is seen that the effect of repeating the dose is more pronounced by the lower concentrations, and is small at the higher dose levels

SUMMARY

Groups of 30 hairless mice were given one single application of methylcholanthrene at different dose levels Other groups of mice were given five repeated applications of the same dose levels of methylcholanthrene

The development of papillomas and carcinomas in the different groups was observed

A single application of 1/64 and 1/32 per cent solution of methylcholanthrene provoked a negligible amount of tumours whereas 1/16

1/8 and 1/1 per cent provoked many tumours in many of the animals A critical dose level seems to exist

Five repeated applications resulted in a higher tumour yield in all groups Only the 1/64 per cent solution provoked relatively few tumours

The significance of these findings are discussed and related to previous reports concerning the tetrazolium test for carcinogenicity

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MORPHOLOGICAL CHANGES IN BOVINE AND HUMAN FIBROBLASTS EXPOSED TO TWO STRAINS OF ROUS SARCOMA VIRUS IN VITRO[1, 2, 3]

By

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In recent years the number of species susceptible to Rous sarcoma virus *in vivo* has been found to include not only fowl but also a variety of mammals (1–13) including monkeys (14). Marked differences between various strains of RSV have been established. *Ahlstrom et al.* comparing $RSV_{Mill\ Hill}$ with $RSV_{Schmidt\ Ruppin}$ found that the former strain induced sarcoma only in chickens whereas the latter gave rise to tumours also in rats, mice, rabbits, hamsters and guinea pigs (1, 2, 4, 13).

Comparatively little is known about the *in vitro* host range of various strains of RSV with the exception of $RSV_{Schmidt\ Ruppin}$ which has been reported to produce round cell foci in rat, mouse and guinea pig cells (4, 15). Recently *Svoboda & Chyle* have described 'malignization' of rat embryonic cells exposed to strain Prague of RSV (16).

The results of exposing bovine and human fibroblasts *in vitro* to two different strains of RSV are given in this report.

MATERIALS AND METHODS

Virus Strains

RSV(SR). For this strain of Rous sarcoma virus we are indebted to Dr. *C. G. Ahlstrom*, Lund, Sweden, who provided us with a sample in 1963. The derivation, history and main biological characteristics of this strain are given in references 1, 2, 4, 6 and 13.

In our laboratory RSV(SR) has been kept in passage in young White Leghorn ...

[1] The following abbreviations have been used: RSV – Rous sar[illegible]om[illegible] RSV(SR ... virus ... associa...

[2] Su... tr...

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Figs 1 2

Fig 1 Uninfected control culture of the bovine lung fibroblast strain B1 Note the regular growth pattern characteristic of normal fibroblasts Unstained culture × 50

Fig 2 Control cells from an unexposed culture of calf fibroblasts Note regular arrangement of elongated cells with monomorphic oval nuclei with several nucleoli Bovine fibroblasts differ from human fibroblasts by the presence of 6 10 rather than 2 4 nucleoli Coverslip culture May Greenwald Giemsa ×670

RSV (EH) This strain of Rous sarcoma virus was obtained from *Engelbreth Holm in 1953*. *Its* history prior to 1953 is not accurately known. It is probable that the virus is derived from the original Rous Chicken Tumor No 1 strain (17) and was kept in Engelbreth Holm's laboratory for about a decade prior to 1953.

The strain gives rise to tumours in chickens morphologically indistinguishable from those caused by the high titre RSV *strain developed by Bryan* (18). After rapid passage through about 20 generations of chicks the yield of RSV (EH) from the tumours is high ($TD_{50} \approx 10^{-6}$). Undiluted virus will cause palpable tumours after 5 days (*Ponten* unpublished).

The RSV (EH) strain was to a limited extent tested *in vivo*.

40 newborn mice of the strain NMRI were injected with minced crude chicken tumour tissue and were observed for 6 months.

18 newborn non inbred rats were also injected with a similar tissue and observed for 3 months.

None of the animals developed tumours *or other lesions during the observation* period. When autopsied at the end of the observation period no pathological lesions were found.

These experimental data are limited but compatible with RSV (EH) belonging to the category of RSV strains with a limited host range *in vivo*.

Preparation of Virus Pools

Tumours were collected 4–5 days after they had become palpable. The tumour tissue was suspended in PBS and homogenized at 23 000 r p m in a VirTis homogenizer in an ice bath for 2 minutes. The suspension was then centrifuged 2 × 20 minutes at 5 000 r p m. Finally the supernatant was filtered through a millipore filter of 0.45 μ pore size. The pools were stored in ampoules at —65° C.

The biological activity of the RSV (EH) and the RSV (SR) pools was tested by wing web *inoculation of samples into week old chicks. With both preparations* 10/10 *birds* developed sarcomas within 9 days.

Tissue Culture Methods

Medium. Eagle's medium (19) with 10 per cent calf serum.

Subcultivation. The cell strains whether infected by virus or not were kept in ... done as soon as the ... 1 four days after each

Exposure to virus. After removal of medium the monolayers were exposed to 0.3 ml of undiluted virus for 1 hour at 37° C. Control cultures were sometimes exposed to PBS but this procedure was omitted later when no differences were demonstrated between control bottles left untreated or exposed to 0.3 ml of PBS.

Incubation. *All cultures were incubated at 37° C. Coverslip preparations* (see below) were incubated in 5 per cent CO_2 in humidified air at 37° C.

Morphological observation. At each subcultivation a coverslip culture was prepared in a small Petri dish. Coverslips were fixed in methanol and stained routinely according to May Greenwald Giemsa. Some cultures were also stained with the Feulgen method.

Cell Strains

Two human (H1 and H2) and two bovine (B1 and B2) strains of fibroblast like cells derived from trypsinized lung tissue were used.

H1 and H2 were developed from a 5 months and 4 months old female foetus respectively. Both were normal and obtained by therapeutical abortion. B1 was developed from a new born calf and B2 from a calf foetus of an estimated age of *2 months*.

EXPERIMENTAL AND RESULTS

Control cultures. From each of the four strains (H1, H2, B1 and B2) five sublines were carried independently. All twenty sublines have been subcultured without difficulty and are at present in passage 20–25





F gs 3–4

F g 3 Ba oj h l c sp ndle cells from stra n H2 of human lung fibroblasts 6 weeks after exposure to RSV (SR Note erv large irregular nucleol Coversl p culture Ma Greenwald G emsa × 1 600

F g 4 Human lung f broblasts of stra n H2 3 veeks after exposure to RSV (SR) Se e al t p cal basoph l c sp ndle cell Near the *upper left* corner a small group of round hyperchromat c cells Note that the altered cells g o on top of the normal l ghtly sta ned f broblasts Co ersl p culture May Greenwald C emsa × 160

Their apparent rate of growth has not changed and they have maintained the same morphology. No signs of any alterations or "transformations" have been observed either in the unstained culture vessels or *in* the stained coverslip preparations removed at each subculture. Spot checks of metaphases have revealed no departure from normal diploidy.

Bovine and human cells derived from trypsinized lung were elongated with pointed ends and had a well-defined homogenous, lightly basophilic cytoplasm and round or oval nuclei of regular form. Variations in the appearance of the individual cells and nuclei were slight. Human cells contained 2–4 small nucleoli in the majority of the cases, whereas *calf cells contained about 6–10 nucleoli*. *Another difference* was that the human cells tended to be more elongated than the bovine cells. The cells grew in regular, slightly curved strands in a monolayer. The appearance of the cultures corresponded to the classical description of fibroblast-like cells *in vitro* (Figs. 1 and 2).

Cultures Exposed to RSV

One subline of H1, three sublines of H2 and three sublines of B1 were exposed to RSV(SR). One subline of H2, one subline of B1 and one subline of B2 were exposed to RSV(EH).

The ten sublines exposed to virus were carried in parallel with the twenty sublines of control cells using the same medium and the same general handling and tissue culture conditions. Sublines of the same strain receiving the same treatment did not differ significantly from each other.

Morphological observations. The alterations in the virus-exposed cultures were complex. To facilitate a description and make comparisons possible, cells were grouped into a few well defined categories. Three types of pathological cells were seen with only a few intermediate forms. The morphology of the three types was similar to that described by *Doljanski* and *Tenenbaum* (21, 22) in avian Rous sarcoma cells in tissue culture.

Basophilic spindle cells. These cells were slightly smaller than normal fibroblasts but retained an elongated shape. A conspicuous alteration was *an intense cytoplasmic basophilia. The nuclei were rounder* than those of normal fibroblasts and contained enlarged nucleoli (Fig. 3). The basophilic spindle cells typically grew on top of the normal fibroblasts forming *easily detectable foci (Fig. 4). Mitosis was rare among* basophilic spindle cells.

Basophilic round cells. These elements had a sparse, well defined and intensely basophilic cytoplasm. *Pedunculated cytoplasmic pseudopodia* were sometimes observed. The nucleus was often eccentrically placed with a coarse chromatin network and prominent nucleoli (Fig. 5 and 6). The diameter of the round cell was slightly larger than the



Figs 3–4

Fig 3 Basophilic spindle cells from strain H2 of human lung fibroblasts 6 weeks after exposure to RSV (SR) Note very large irregular nucleoli Coverslip culture May Greenwald Giemsa × 1 600

Fig 4

Giemsa × 160

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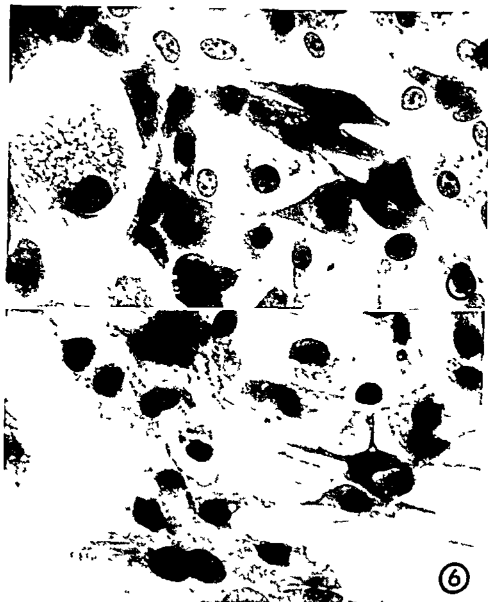
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F g 4 Human lung f broblasts of strain H2 3 weeks after exposure to RSV (SR)



Figs 5–6

Fig 5 Altered cells of different types from strain B1 of bovine lung fibroblasts 3 weeks after exposure to RSV (SR) To the *left* a vacuolated giant cell Most of the cells are basophilic round cells of typical appearance with sparse dark cytoplasm and round often eccentrically placed nuclei with large nucleoli A group of three parallel basophilic spindle cells can be seen near the *centre* of the picture Coverslip culture May Greenwald Giemsa × 620

Fig 6 Human lung fibroblasts of strain B2 4 weeks after exposure to RSV (SR) High magnification of a focus of altered cells Note the general similarity to the altered calf cells of Fig 5 The field is dominated by basophilic round cells Near the *bottom* a bi nucleated giant cell showing a mirror image type of symmetry Most nuclei show clearly enlarged nucleoli Coverslip culture May Greenwald Giemsa × 620



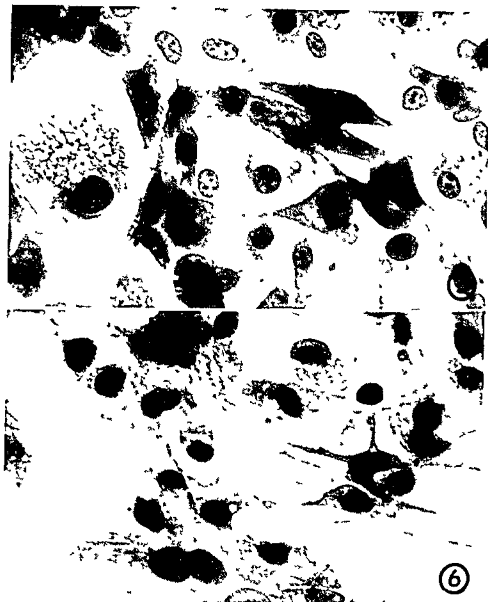
Figs 7 9

Fig 7 Calf fibroblasts 9 weeks after exposure to RSV (EH) The only recognizable change was the occurrence of scattered vacuolated *giant cells* Due to a fixation artefact they have shrunk from the surrounding normal fibroblasts

Fig 8

× 670

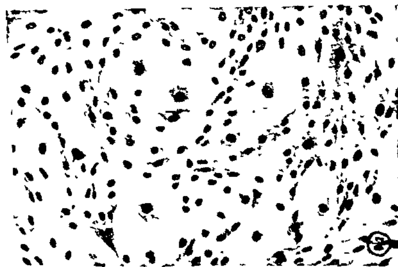
Fig 9 Same culture as in Fig 9 High magnification of a binucleated "mirror image" cell Coverslip culture × 1 600



Figs 5–6

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Figs 7–9

Fig 7 Calf fibroblasts 9 weeks after exposure to RSV (EH) The only recognizable change was the occurrence of scattered vacuolated giant cells Due to a [illegible] culture May Grunwald Giemsa × 160

Fig 8 Calf fibroblasts of strain B1 10 weeks after exposure to RSV (SR) Two binucleated 'mirror image' cells of different size are seen Coverslip culture × 670

Fig 9 Same culture as in Fig 9 High magnification of a binucleated "mirror image" cell Coverslip culture × 1 600

TABLE 1

Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B1 Exposed to RSV Schmidt Ruppin (RSV (SR)) and RSV Engelbreth Holm (RSV (EH))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11 12
RSV(SR)	Basophilic spindle cells	—	—	+	++	++	+++	++++	++++	++++
	Basophilic round cells	—	—	+	+	+	++	+++	++++	++++
	Vacuolated giant cells	—	—	+	+	+	+	+	+++	+++
RSV(FH)	Basophilic spindle cells	—	—	—	—	—	—	—	—	—
	Basophilic round cells	—	—	—	—	—	—	—	—	—
	Vacuolated giant cells	—	—	—	++	+	++	+	+	+

\+ = Scattered pathological cells in culture Thorough examination of whole culture necessary to detect changes
++ = Pathological cells in every 3rd 4th field of vision (appr corresponding to 3 5 per cent pathological cells)
+++ = Pathological cells almost in every field of vision (appr corresponding to 20 per cent of pathological cells)
++++ = In every field of vision numerous pathological cells (appr corresponding 40 per cent pathological cells)

TABLE 2

Time Sequence of Morphological Changes in Human Cell Cultures of Strain H1 Exposed to RSV Schmidt-Ruppin (RSV(SR))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8 10	11 12
RSV (SR)	Basophilic spindle cells	—	—	—	+	+	+	—	—	—
	Basophilic round cells	—	—	—	—	—	+	—	—	—
	Vacuolated giant cells	—	—	—	—	—	+	—	—	—

\+ = Scattered pathological cells in culture Thorough examination of whole culture necessary to detect changes
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++++ = In every field of vision numerous pathological cells (appr corresponding 40 per cent pathological cells)

TABLE 3

Time Sequence of Morphological Changes in Human Cell Cultures of strain H2 Exposed to RSV Schmidt Ruppin (RSV (SR)) and RSV Engelbreth Holm (RSV (EH))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11-12
RSV (SR)	Basophilic spindle cells		—	+	+	++	++	++	+	+
	Basophilic round cells			+	+	+	—	—	—	—
	Vacuolated giant cells	—	—	—	+	+	—	—	—	—
RSV (EH)	Basophilic spindle cells	—	—	—	—	—	—		*	
	Basophilic round cells	-	—	—	—	—	—	—		
	Vacuolated giant cells	—	—	—	—	—	—	—		

* The H2 cells were exposed to RSV (EH) 5 weeks later than to RSV(SR)

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++++ — In every field of vision numerous pathological cells (appr. corresponding 40 per cent pathological cells)

TABLE 4

Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B2 Exposed to RSV Engelbreth Holm (RSV (EH))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11-12
RSV(EH)	Basophilic spindle cells	—	—	—	—	—	—	—	—	—
	Basophilic round cells	—	—	—	—	—	-	—	—	—
	Vacuolated giant cells	—	—	—	+	++	++	++	++	++

\+ — Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes

++ — Pathological cells in every 3rd-4th field of vision (appr. corresponding to 3-5 per cent pathological cells)

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++++ — In every field of vision numerous pathological cells (appr. corresponding 40 per cent pathological cells)

TABLE 1

Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B1 Exposed to RSV Schmidt Ruppin (RSV (SR)) and RSV Engelbreth Holm (RSV (EH))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11 12
RSV(SR)	Basophilic spindle cells	—	—	+	++	++	+++	++++	++++	++++
	Basophilic round cells	—	—	+	+	+	++	+++	++++	++++
	Vacuolated giant cells	—	—	+	+	+	+	+	+++	+++
RSV (EH)	Basophilic spindle cells	—	—	—	—	—	—	—	—	—
	Basophilic round cells	—	—	—	—	—	—	—	—	—
	Vacuolated giant cells	—	—	—	++	+	++	+	+	+

\+ = Scattered pathological cells in culture Thorough examination of whole culture necessary to detect changes
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Time Sequence of Morphological Changes in Human Cell Cultures of Strain H1 Exposed to RSV Schmidt Ruppin (RSV (SR))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11 12
RSV (SR)	Basophilic spindle cells	—	—	—	+	+	+	—	—	—
	Basophilic round cells	—	—	—	—	—	+	—	—	—
	Vacuolated giant cells	—		—	—	—	+	—	—	—

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TABLE 3

Time Sequence of Morphological Changes in Human Cell Cultures of strain H2 Exposed to RSV Schmidt Ruppin (RSV (SR)) and RSV Engelbreth Holm (RSV (FH))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8 10	11 12
RSV (SR)	Basophilic spindle cells	—	—	+	+	++	++	++	+	+
	Basophilic round cells	—	—	+	+	+	—	—	—	—
	Vacuolated giant cells	—	—	—	+	+	—	—	—	—
RSV (FH)	Basophilic spindle cells	—	—	—	—	—	—	—	*	
	Basophilic round cells	—	—	—	—	—	—	—		
	Vacuolated giant cells	—	—	—	—	—	—	—		

* The H2 cells were exposed to RSV(FH) 5 weeks later than to RSV (SR)

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++++ — In every field of vision numerous pathological cells (appr corresponding 40 per cent pathological cells)

TABLE 4

Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B2 Exposed to RSV Engelbreth-Holm (RSV (FH))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11 12
RSV (FH)	Basophilic spindle cells	—	—	—	—	—	—	—	—	—
	Basophilic round cells	—	—	—	—	—	—	—	—	—
	Vacuolated giant cells	—	—	—	+	++	++	++	++	++

+ — Scattered pathological cells in culture Thorough examination of whole culture necessary to detect changes
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TABLE 1

Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B1 Exposed to RSV Schmidt-Ruppin (RSV (SR)) and RSV Engelbreth-Holm (RSV (EH))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11-12
RSV(SR)	Basophilic spindle cells	—	—	+	++	++	+++	++++	++++	++++
	Basophilic round cells	—	—	+	+	+	++	+++	++++	++++
	Vacuolated giant cells	—	—	+	+	+	+	+	+++	+++
RSV (EH)	Basophilic spindle cells	—	—	—	—	—	—	—	—	—
	Basophilic round cells	—	—	—	—	—	—	—	—	—
	Vacuolated giant cells	—	—	—	++	+	++	+	+	+

+ = Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes
++ = *Pathological* cells in every 3rd-4th field of vision (appr. corresponding to 3-5 per cent pathological cells)
+++ = Pathological cells almost in every field of vision (appr. corresponding to 20 per cent of pathological cells)
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TABLE 2

Time Sequence of Morphological Changes in Human Cell Cultures of Strain H1 Exposed to RSV Schmidt-Ruppin (RSV (SR))

Virus	Cell type	Time in weeks after exposure to virus								
		1	2	3	4	5	6	7	8-10	11-12
RSV (SR)	Basophilic spindle cells	—	—	—	+	+	+	—	—	—
	Basophilic round cells	—	—	—	—	—	+	—	—	—
	Vacuolated giant cells	—	—	—	—	—	+	—	—	—

+ = Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes
++ = Pathological cells in every 3rd-4th field of vision (appr. corresponding to 3-5 per cent pathological cells)
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TABLE 3

T e Seq e e f Morphologe l Cha ges n H an Cell Cult res of stra n H2 Exposel to RSV Sel n lt Rutt (RSV (SR)) a l RSV Eng lbrell Holn (RSV (EH))

Virus	Ce l type	Time in w ks after expos e to r s								
		1	2	3	4	5	6	7	8 10	11 12
RSV (SR)	Bas p lilic 1 in lle cells	—	—	+	+	++	++	++	+	+
	Basop lile r und c lls			+	+	+	—	—	—	—
	Vac late l giant cells		—		+	+	—	—	—	—
RSV (EH)	Ba ophilic spindle cells	—	—	—		—	—			
	Basophilic round cells	—		—	—	—	—	—		
	Vacuolated giant cells		—	—	—	—	—	—		

The H2 cells vere exposel to RSV (EH) 5 veel s later than to RSV (SR)

+ — Scattered pathological cells in c lt re Th rough examinat on of v h le cult ire nece ary to letect changes
++ — Pathological cells in e er 3r l 4th f el l of v sion (appr corre pon ling t 3 5 per cent path l gical cells)
+++ — Pathological cells almost in every fiel l of vision (appr c rresp n ling to 20 p r cnt f pat ol g cal cells)
++++ — In every fiel l of visi n n merous path ol g cal cells (appr corre p nd ng 40 per cent patl l gical cells)

TABLE 4

T n e Seq ence of Morpholog cal Cl a ges i Bo ne Cell Cultures of Stra B2 Expos l to RSV Engelbreth Hol n (RSV (EH))

Vir s	C ll ype	Time in weeks af er expos re to i s								
		1	2	3	4	5	6	7	8-10	11 12
RSV (EH)	Basop l ilic spin lle cells	—	—	—	—	—	—	—	—	—
	Basophilic r und cells	—	—	—	—	—		—	—	—
	Vacuolated giant cells	—	—	—	+	++	++	++	++	++

+ — Scatt red path olog cal cells in cult re Thoro gh examination of wh le culture ne essar t let t hang s
++ — Pathol gical cells n e ery 3r l 4tl fiel l of v sio (appr c rr pon ling to 3 5 per cent patl l gical ells)
+++ — Pathol gical ells alm st in every f el l f vision (appr c rresp on ling to 20 per ce t of path l gical cells)
++++ — I every fiel l of vision n imerous pathol gical cells (appr corresp n ling 40 per cent patl ol gical cells)

width of the normal fibroblasts These cells occurred either in foci or diffusely scattered over the culture They tended to detach easily Mitosis was rare

Vacuolated giant cells (Figs 7, 8 and 9) These cells showed great variations in size from approximately the size of a normal fibroblast to monster cells approaching 0 5 mm in diameter The cytoplasm usually contained numerous vacuoles of different size In some cells a perinuclear arrangement of the vacuoles was found, in others they were diffusely scattered The cytoplasm showed variable degree of basophilia, which, however, rarely was as intense at that of the previous two cell types The nuclei were large and often lobulated and in many cells several large nucleoli were seen Many bi- and tetranucleated cells showed a symmetric arrangement of the nuclei (Figs 8 and 9)

The sequence of the appearance of pathological cells in bovine and human cultures exposed to RSV(SR) and RSV(EH) is given in Tables 1–4

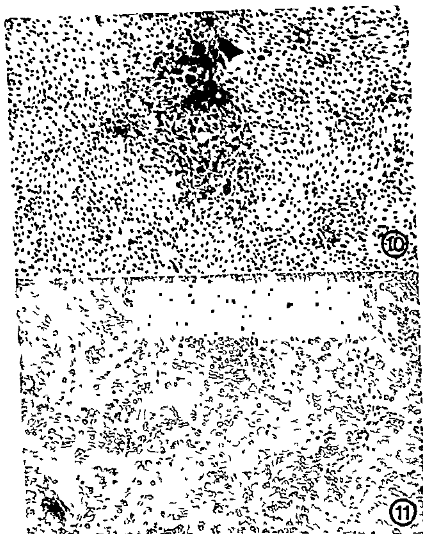
RSV Schmidt-Ruppin Series

Three weeks after exposure, the three sublines of the B1 cell strain of calf fibroblasts exposed to RSV(SR) began to show foci of basophilic spindle and round cells, which increased rapidly in number (Table 1) At the same time a moderate amount of vacuolated giant cells appeared By 8 weeks the cultures seemed to have reached a stable state characterized by the presence of a mixture of both morphologically normal fibroblasts and basophilic spindle or round cells and vacuolated giant cells The altered cells tended to occur in foci (Fig 10) Particularly, the basophilic cells tended to grow on top of other cells forming irregular sheets or piles (Figs 11, 12 and 13) In spite of the profound morphological alterations, the over-all growth rate of the cultures did not change materially

The subline of the H1 cell strain (human lung fibroblasts) exposed to RSV(SR) showed the following changes

Four to six weeks after infection a small number of foci consisting of a few basophilic spindle cells were observed (Table 2) In the six-week-samples a small number of basophilic round cells and vacuolated giant cells was also seen In contrast to the cultures of bovine cells, the altered human cells of strain H1 disappeared rapidly so that cover slips 7 weeks or later after exposure to virus were morphologically normal

The three sublines of the H2 human lung fibroblast strain exposed to RSV(SR) began to show foci of basophilic spindle and round cells three weeks after exposure to virus (Table 3) A small number of such foci were still seen at the time of writing *i e* 12 weeks after exposure Basophilic round cells and vacuolated giant cells were seen only during a few weeks in the beginning of the phase when the cultures started to change morphologically



Figs 10 11

Fig 10 Strain B1 of bovine lung fibroblasts 3 weeks after exposure to RSV (SR) Typical focus of altered cells sharply distinguished from surrounding fibroblasts of normal appearance Coverslip culture May Greenwald Giemsa × 50

Fig 11 Bovine lung fibroblasts of strain B1 7 weeks after exposure to RSV (SR) Note large numbers of abnormal cells, most of which are highly refractile cells corresponding to the "basophilic round cells" (Table 1) Unstained culture × 50

width of the normal fibroblasts These cells occurred either in foci or diffusely scattered over the culture They tended to detach easily Mitosis was rare

Vacuolated giant cells (Figs 7, 8 and 9) These cells showed great variations in size from approximately the size of a normal fibroblast to monster cells approaching 0 5 mm in diameter The cytoplasm usually contained numerous vacuoles of different size In some cells a perinuclear arrangement of the vacuoles was found, in others they were diffusely scattered The cytoplasm showed variable degree of basophilia, which, however, rarely was as intense at that of the previous two cell types The nuclei were large and often lobulated and in many cells several large nucleoli were seen Many bi- and tetranucleated cells showed a symmetric arrangement of the nuclei (Figs 8 and 9)

The sequence of the appearance of pathological cells in bovine and human cultures exposed to RSV(SR) and RSV(EH) is given in Tables 1–4

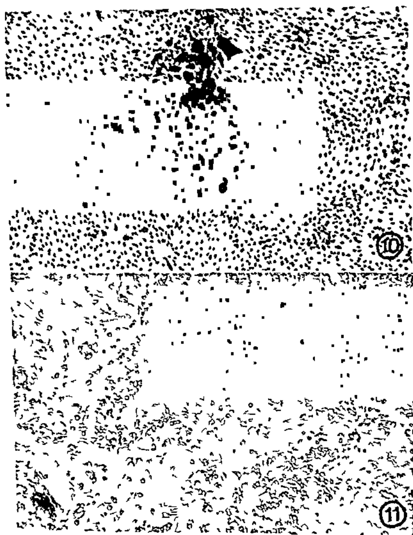
RSV Schmidt-Ruppin Series

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The three sublines of the H2 human lung fibroblast strain exposed to RSV(SR) began to show foci of basophilic spindle and round cells three weeks after exposure to virus (Table 3) A small number of such foci were still seen at the time of writing i e 12 weeks after exposure Basophilic round cells and vacuolated giant cells were seen only during a few weeks in the beginning of the phase when the cultures started to change morphologically



Figs 10 11

Fig 10 Strain B1 f bovine lung fibrol lasts 3 weeks after exposure to RSV (SR) Typical focus of altered cells sharply distinguished from surrounding fibro blasts of normal appearance Coverslip culture May Greenwald Giemsa × 50

Fig 11 B vine lung fil roblasts of strain B1 7 weeks after exposure to RSV (SR) Note large numl ers of abnormal cells most of which are highly refractile cells corresponding to the basophilic round cells" (Table 1) Unstained culture × 50



Figs 12–13

Fig 12 Bovine lung fibroblasts of strain B1 7 weeks after exposure to RSV (SR) High magnification of an accumulation of the round highly refractile cells characteristic of the RSV (SR) effect Unstained culture × 160

Fig 13 Calf fibroblasts of strain B1 10 weeks after exposure to RSV (SR) Highly abnormal culture with a mixture of cells characteristic of the balanced state reached about 8 weeks after exposure to virus Normally appearing cells are intermingled with basophilic darkly stained cells of different shapes which tend to accumulate in small heaps Coverslip culture May Greenwald Giemsa × 160

Both human cell strains showed no difference in their over-all growth rate compared to uninfected parallel control cultures

RSV Engelbreth Holm Series

The two bovine cell strains B1 (Table 1) and B2 (Table 4) exposed to RSV(EH) showed similar responses Vacuolated giant cells appeared 4 weeks after infection and have persisted for 8 weeks at the time of writing

In contrast to the effects caused by RSV(SR) no basophilic spindle or round cells were observed in the cultures exposed to RSV(EH)

One subline of the human strain H2 exposed to RSV(EH) has not shown any abnormalities during a seven week period of observation at the time of writing

DISCUSSION

Human and bovine cells exposed to the Schmidt-Ruppin strain of RSV responded with cytological alterations similar to those previously described in RSV infected chicken (21, 22, 26) or murine (15, 16) cells The changes were complex but three distinct types of pathological elements (basophilic spindle cells, basophilic round cells and vacuo lated giant cells) could be discerned This similarity between changes in avian and murine cells on the one side and bovine and human cells on the other, indicates that the same basic phenomena may be evoked in the four systems by exposure to at least certain strains of RSV

A wide host range comparable to that of RSV(SR) including cells of such a zoologically distant origin as avian, murine, bovine and primate tissues has not been demonstrated for other tumour viruses It indicates that the RSV(SR) strain is endowed with a property that would make extensive spread in nature possible

Cytologically the RSV induced alterations differed in a characteristic way from morphological changes induced by other tumour viruses (27, 28, 29, 30) With polyoma virus in mouse and hamster cell cul tures a pronounced "criss-cross' growth pattern seems to be the most characteristic feature (27) SV 40 transformation on the other hand seems to involve a lack of "inhibition of mitosis" and the appearance of epithelial like cells as a primary change (28, 29, 30)

Another difference was the failure of RSV altered cells to 'breed true In human cell cultures the proportion of altered cells decreased on prolonged serial cultivation In the bovine system they remained at an approximately stable level for a long period and were still mixed with cytologically normal fibroblasts even after 12 weeks of observa- tion The mechanism responsible for the maintenance of this balance is unknown It may be that altered cells have no select

ed cells may then be counteracted by a slow cytopathogenicity The last type of growth mechanism is principally the same as that proposed for Rous sarcoma transplanted by cells in an isologous *in vivo* system (31)

Two differences in the activity of RSV(SR) and RSV(EH) were observed The latter strain failed to alter human fibroblasts whereas the former evoked changes in the same system In calf fibroblasts both strains gave rise to alterations The difference between the two virus strains in their effects on calf fibroblasts was qualitative, since cultures exposed to RSV(SR) displayed the complete spectrum of morphological changes, whereas calf fibroblasts exposed to RSV(LH) only showed vacuolated giant cells but no basophilic spindle or round cells We do not want to emphasize the importance of the latter difference between the two virus strains, since we have observed a full spectrum of alterations in bovine kidney cultures exposed to RSV(EH) (unpublished)

Another reason to avoid premature conclusions concerning the activity of different RSV preparations is the demonstration by *Rubin* and coworkers (23, 24) of an intimate functional relationship between such members of the avian tumour virus complex as RSV, RIF and RAV Crude tumour filtrates were used in the present work and it is not known whether the effects observed represent the action of one or several different viruses belonging to the avian tumour virus complex

SUMMARY

Human and bovine fibroblast-like cells cultured from lung have been exposed to Rous sarcoma virus *in vitro* The Schmidt-Ruppin and the Engelbreth-Holm strains of RSV were used

With the Schmidt Ruppin strain human and bovine tissue cultures responded with cellular morphological alterations similar to previously described changes in chicken and murine cells exposed to RSV Basophilic spindle and round cells as well as vacuolated giant cells appeared between 3–6 weeks after exposure to virus These abnormal cells appeared in greater proportion in bovine than in human fibroblast cultures In the bovine cultures the pathological cells reached a stable state with about 40 per cent abnormal cells in the cultures In the human cultures the pathological cells increased in number initially With time the proportion of morphologically altered cells decreased and in one strain they seemed to disappear altogether

The Engelbreth Holm strain of RSV did not evoke any alterations in human cells In calf cell cultures vacuolated giant cells but no basophilic spindle or round cells were noted

Control cultures carried under the same conditions *in vitro* as the cultures exposed to RSV displayed no morphological alterations

It is concluded that the Schmidt-Ruppin strain of RSV is able to alter human and bovine cells in a way cytologically indistinguishable from the manner in which chicken and rat cells have been reported to "transform" after exposure to RSV

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ed cells may then be counteracted by a slow cytopathogenicity. The last type of growth mechanism is principally the same as that proposed for Rous sarcoma transplanted by cells in an isologous *in vivo* system (31).

Two differences in the activity of RSV(SR) and RSV(EH) were observed. The latter strain failed to alter human fibroblasts whereas the former evoked changes in the same system. In calf fibroblasts both strains gave rise to alterations. The difference between the two virus strains in their effects on calf fibroblasts was qualitative, since cultures exposed to RSV(SR) displayed the complete spectrum of morphological changes, whereas calf fibroblasts exposed to RSV(LH) only showed vacuolated giant cells but no basophilic spindle or round cells. We do not want to emphasize the importance of the latter difference between the two virus strains, since we have observed a full spectrum of alterations in bovine kidney cultures exposed to RSV(EH) (unpublished).

Another reason to avoid premature conclusions concerning the activity of different RSV preparations is the demonstration by *Rubin* and coworkers (23, 24) of an intimate functional relationship between such members of the avian tumour virus complex as RSV, RIF and RAV. Crude tumour filtrates were used in the present work and it is not known whether the effects observed represent the action of one or several different viruses belonging to the avian tumour virus complex.

SUMMARY

Human and bovine fibroblast-like cells cultured from lung have been exposed to Rous sarcoma virus *in vitro*. The Schmidt Ruppin and the Engelbreth-Holm strains of RSV were used.

With the Schmidt-Ruppin strain human and bovine tissue cultures responded with cellular morphological alterations similar to previously described changes in chicken and murine cells exposed to RSV. Basophilic spindle and round cells as well as vacuolated giant cells appeared between 3–6 weeks after exposure to virus. These abnormal cells appeared in greater proportion in bovine than in human fibroblast cultures. In the bovine cultures the pathological cells reached a stable state with about 40 per cent abnormal cells in the cultures. In the human cultures the pathological cells increased in number initially. With time the proportion of morphologically altered cells decreased and in one strain they seemed to disappear altogether.

The Engelbreth Holm strain of RSV did not evoke any alterations in human cells. In calf cell cultures vacuolated giant cells but no basophilic spindle or round cells were noted.

Control cultures carried under the same conditions *in vitro* as the cultures exposed to RSV displayed no morphological alterations.

It is concluded that the Schmidt Ruppin strain of RSV is able to alter human and bovine cells in a way cytologically indistinguishable from the manner in which chicken and rat cells have been reported to "transform" after exposure to RSV

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The Enterovirus Department Statens Seruminstitut Copenhagen Denmark

ANTIBODY RESPONSE TO TYPE 1 ORAL POLIO VACCINE

By

INGER PETERSEN and HERDIS VON MAGNUS

Received 4 ix 64

In April–May 1963 a vaccination campaign with oral polio vaccine type 1 was carried out in Denmark (1,8) In connection with this program, a study of the antibody formation in 297 individuals was performed The present paper records the results of this study

MATERIALS AND METHODS

Vaccine Monovalent type 1 oral poliovirus vaccine prepared from the Sabin strain LSc 2ab by the Connaught Laboratories Toronto Canada was used The vaccine was bought concentrated and was diluted in a mixture of equal amounts of 2 molar NaCl [illegible]

to room temperature for 3 hours before actual use

Each person was given 3 drops of vaccine on a lump of sugar Young children received the vaccine in 30 per cent syrupus sacchari on a disposable plastic spoon

Collection of serum Blood samples for antibody titrations were obtained before and 5–7 weeks after the vaccination from 297 persons aged 5 months to 68 years [1]

Antibody titrations Paired serum samples from all 297 persons were titrated for type 1 polio virus neutralizing antibodies In addition the sera from 135 of these individuals (selected at random) were titrated for type 2 and 3 antibodies

The virus strains employed in the neutralization tests were type 1 Brunhilde [illegible]

[illegible] the virus serum mixture was inoculated into each of two tissue culture tubes with a confluent monolayer of primary monkey kidney cells and 1 8 ml of medium 199 The tubes were placed in roller drums or stationary racks (usually roller drums for the first 3 days after seeding and thereafter in stationary racks) The tubes were [illegible]

us [illegible]

al [illegible]

10 [illegible] and a titration of a local reference serum

Some tests also comprised a titration of the WHO International Reference Preparations for anti poliomyelitis serum types 1 2 and 3 From 22 titrations of each type of serum it was found that the geometric mean titre of the Int Ref Serum type 1 was 90 type 2 had a titre of 150 and type 3 a titre of 60

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[1] The sera were [illegible] mann [illegible] vangean [illegible] institutio [illegible] St Tulin [illegible]

25 *Dulbecco R* & *Vogt M* Plaque formation and isolation of pure lines with poliomyelitis viruses J Exp Med *99* 167 181 1954

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in about one fourth of the individuals while the majority showed no change in antibody titre

TABLE 1

Type 1 Conversion Rate in Various Age Groups after Oral Type 1 Vaccination

Age	No	Type 1 negative				Conversion rate
		Before	After	Before	After	
<1	12	5 (7)*	2			
1	27	9	–	17/49	3/49	14/17 = 82 %
2	9	0 (1)†	1			
3	1	0	–			
4	12	3	–			
5	1	1	–			
6	17	8	–			
7	12	4	–	38/100	0/100	38/38 = 100 %
8	17	7	–			
9	14	5	–			
10	12	4				
11	15	6				
12	12	1				
13	13	0	–			
14	14	0	–			
15	9	2	1			
16	4	0		4/69	2/69	2/4 (= 50 %)
17	3	0				
18	4	1	1			
19	6	0	–			
20	4	0				
21–30	20	1	–			
31–40	18	0	–	2/79	1/79	1/2 (= 50 %)
>40	41	1	1			
	297	58 (61)	6			

* 2 children had a maternal antibody titre of 6 before vaccination (see Table 3)
† This child had an antibody titre of 8 before vaccination and no antibodies after vaccination

In Table 1 the conversion rate from type 1 negative to type 1 positive is shown for the various age groups included in the study. It will be seen that in the age group under 4 years, 14 out of 17 type 1-negat[illegible]

[illegible] type 1 antibodies after the oral vaccination

In the age group 4–11 years, all 38 children without type 1 antibodies before vaccination responded to the oral vaccine with formation of type 1 neutralizing antibodies, the conversion rate thus being 100 per cent

In the age groups of 12 years and older only 6 out of 148 individuals lacked type 1 antibodies before vaccination. Three of these 6 types 1

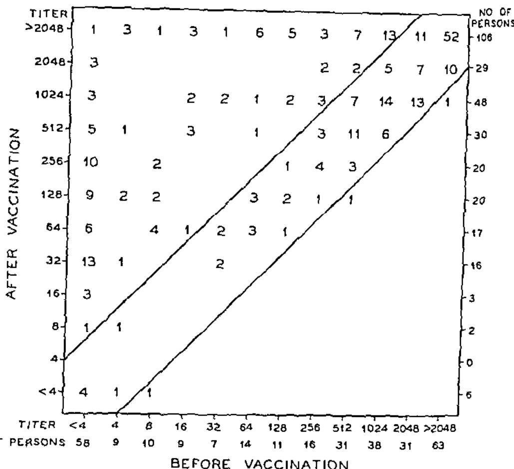


Fig 1

Type 1 antibody response of 297 persons aged 5 months–68 years after feeding of monovalent type 1 Sabin vaccine

RESULTS

Type 1 antibody response Fig 1 shows the type 1 neutralizing antibody titres before and 5–7 weeks after vaccination It will be seen that the homologous antibody response was very satisfactory Of 58 individuals without type 1 antibodies before vaccination, only 4 remained negative after vaccination, and the great majority showed antibody titres of 32 or higher, the geometric mean titre being 120

Of the 28 individuals with antibody titres of 4 16 before vaccination, 25 showed an increase in antibody level with titres ranging from 32 to >2048 after vaccination The remaining 3 persons with low antibody levels, however, did not react to the vaccination and two of them (a 6 months old child without previous Salk vaccination and a 2 year-old child who had received the 4th Salk vaccination 1 year before) showed an antibody titre of <4 after vaccination

When the pre vaccinal antibody level was higher i e more than 32, the picture was different in that an antibody rise was demonstrated only

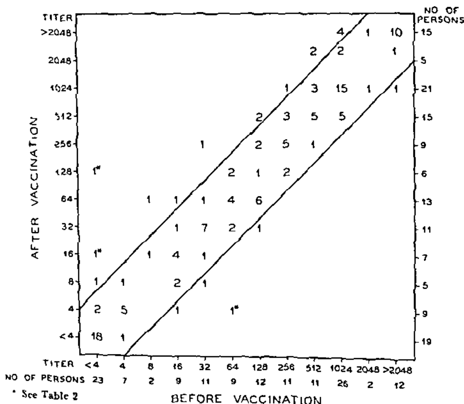


Fig 3

Type 3 antibody response of 135 persons aged 5 months–68 years after feeding of monovalent type 1 Sabin vaccine

group has been recorded This figure shows the effect of the oral *type 1 vaccination*, which is particularly striking in the age group 4–11 years

In Fig 5 the geometric mean of type 1 antibody titres *before* and *after* vaccination has been correlated with the age of the individuals *It will be seen that the* geometric mean titre after vaccination ranged from 300 to 1700 for all age groups except for the very youngest under one year, who had a mean antibody titre of 114

Heterologous Antibody Response 135 of the 297 paired sera were selected at random for study of type 2 and type 3 neutralizing antibodies

Fig 2 shows that a number of individuals had a moderate heterologous type 2 antibody response to the oral type 1 vaccine In 14 persons the rise in titre was more than 4-fold A 4 fold change in titre is within the experimental error Since, however, twelve persons showed a 4 fold rise while only 3 individuals showed a *4-fold decrease* in titre, this finding seems to indicate a slight heterologous response also in some of these individuals

In Fig 3 the type 3 antibody titres are recorded, and it *is evident*

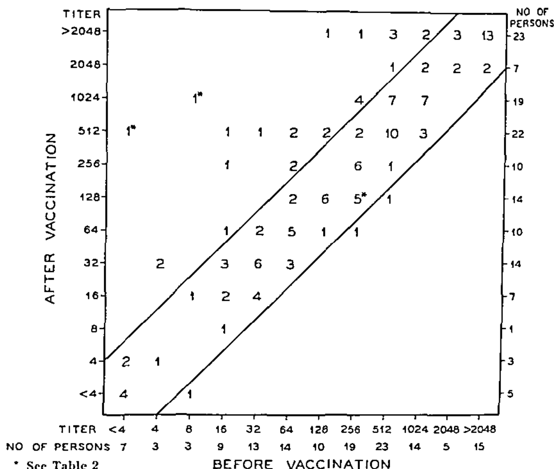


Fig 2

Type 2 antibody response of 135 persons aged 5 months–68 years after feeding of monovalent type 1 Sabin vaccine

negative persons responded to the vaccination, and only 3/148 = 2 per cent were without type 1 antibodies after the oral vaccination

In Table 3, the type 1 antibody response in 12 children under one year of age is listed individually together with their Salk vaccination status Nine of these children responded to the vaccination with good levels of type 1 antibodies, while the result for the remaining 3 children was unsatisfactory Child No 8909, 5 months old, did not have antibodies neither before nor after feeding of the type 1 vaccine A second child, No 11042, showed a low level of maternal antibodies at the time of vaccination, and the antibody titre dropped from 6 to <4 during the following 5 weeks A third child, No 11046, had received 3 Salk vaccinations and had a type 1 antibody titre of 90 This child did not have a booster response after feeding of type 1 vaccine, but showed a titre of 64 five weeks after vaccination

In Fig 4 the 297 individuals have been divided into groups according to age, and the percentage of individuals with type 1 antibodies before and after vaccination as well as the number of persons in each age

Fig 4

Polio type 1 antibody pattern in various age groups before and after oral type 1 vaccination

Fig 5

Geometric mean of polio type 1 antibody titers in various age groups before and after oral type 1 vaccination

that the heterologous type 3 antibody response was less pronounced than the type 2 response

Among the 135 persons studied three showed an unusual heterologous antibody reaction The serum titres for these 3 individuals are in Figs 2 and 3 marked with an asteric and they have also been listed separately in Table 2 The 2 first subjects (Nos 8708 and 8702) both aged 6 years show very pronounced heterologous antibody response, in spite of the fact that 8708 was triple negative before vaccination and 8702 had only type 2 pre-vaccinal antibodies The third person, No 8969, 10 years old, very unexpectedly show a 16-fold drop in type 3 antibodies in relation to type 1 oral polio vaccination It seems very unlikely that these irregular findings can be real Probably a mislabelling of the sera has taken place

TABLE 2

3 Children with Unusual Heterologous Antibody Response to Type 1 Oral Polio Vaccination

No	Age in years	Antibody titres					
		Before vacc			After vacc		
		Type			Type		
		1	2	3	1	2	3
8708	6	0*	0	0	360	720	23
8702	6	0	11	0	720	1450	180
8968	10	0	256	64	45	128	4

* < 4 is recorded as 0

TABLE 3

Polio Virus Type 1 Antibodies before and after Oral Type 1 Polio Vaccination in Children Aged 5 11 Months

Lt nr	Age in months	Previous Salk vacc	Antibody titre against type 1	
			Before oral vacc	After oral vacc
11001	5	0	6	128
8909	5	0	0	0
11044	6	0	0	180
8908	6	0	0	128
11043	6	0	64	180
11042	6	0	6	0
11041	6	0	0	45
11003	7	2	90	>2048
8904	8	0	0	256
11046	10	3	90	64
11002	10	3	180	>2048
11047	11	3	32	1450

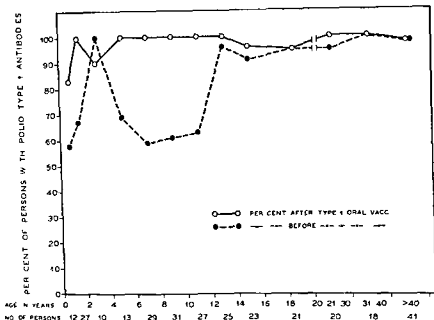


Fig 4

Polio type 1 antibody pattern in various age groups before and after oral type 1 vaccination

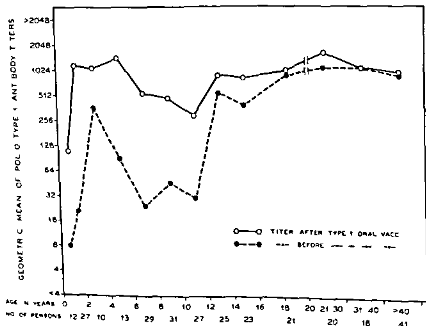


Fig 5

Geometric mean of polio type 1 antibody titers in various age groups before and after oral type 1 vaccination

DISCUSSION

It is generally accepted that the use of inactivated polio vaccine of the Salk type has had a very pronounced preventive effect on the occurrence of paralytic polio in many parts of the world (3). However, it is known also that the vaccination antibodies decrease significantly already after a few years (4, 5, 6, 7, 11).

Oral polio virus vaccine has been widely used both in unvaccinated and in solidly immunized populations. Both monovalent, bivalent, and trivalent vaccines have been employed and they have all been found to produce antibody formation in a high percentage of the vaccinated individuals provided that a proper virus dose was used (3).

In the present study paired sera from individuals receiving a dose of $10^{5.3}$ TCID$_{50}$ monovalent, oral type 1 polio vaccine (*Sabin*) were studied in neutralization tests. The material comprised 297 individuals between 5 months and 68 years the great majority of whom previously had received 3 or 4 vaccinations with inactivated polio vaccine (*Salk*). It was found that there was a good response to the single dose of oral vaccine. Before the vaccination 58 of the 297 individuals or 20 per cent lacked demonstrable type 1 antibodies while only 6 persons or 2 per cent had no antibodies after vaccination (Table 1). Individuals in the age group 4–11 years who before the vaccination showed the largest percentage of sero-negatives, 38 per cent, reacted particularly well in that all individuals developed type 1 antibodies as a result of the oral vaccination. These findings correspond well with findings in other studies (3, 11, 13).

The antibody levels were also satisfactory. The geometric mean titre of neutralizing antibodies reached 120 in the group of individuals who did not have type 1 antibodies before vaccination. In the neutralization tests the sera were tested against Brunhilde virus. If LSc had been used, the serum titre would have been slightly higher (12).

In accordance with the results obtained by others (9, 10) it was found that the type 1 oral vaccine elicited a moderate heterotypic type 2 antibody response (Fig. 2). A slight heterotypic type 3 response was noted in some individuals (Fig. 3).

SUMMARY

A dose of $10^{5.3}$ TCID$_{50}$ of Sabin type 1 polio vaccine was given to 297 individuals between the age of 5 months and 68 years. The antibody response was very satisfactory. Of 58 persons (20 per cent) without type 1 antibodies before vaccination, only 6 (2 per cent) remained negative, and the great majority of the type 1 sero negative individuals reached antibody levels of 32 or higher, the geometric mean titre being 120. The few non responding individuals were partly found in the older age groups and partly in children of 5 months–3 years. In the age group 4–11 years, in which no less than 38 per cent of the individuals

had no type 1 antibodies before the oral vaccination all the sero negative children showed type 1 antibodies after vaccination

A total of 135 persons were studied for heterotypic antibody formation and it was found that the type 2 response was moderate while the type 3 response was less pronounced

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Acta path et microbiol scandinav 62, 340-348, 1964

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BIOLOGICAL ACTIONS OF POLYANIONS

A Comparative Study of the Effect on Herpes Virus, Blood Coagulation, Red Blood Cells and Growth Behaviour of Cells on a Glass Surface

By

ANTTI VAHERI, EERO IKKALA, ERKKI SAXEN and KARI PENTTINEN

Received 31 iii 64

A number of reports have been published on the activities of polyanions in most different biological systems Various enzymes (*Diczfalusy et al* 1953, *Dierick & Stockx* 1957, *Heymann et al* 1958 and 1959), viruses (*Neher & Krafolder* 1955, *Penttinen* 1956, *Stahmann & Gothoskar* 1958, *Young & Mora* 1960) and tumour growth (*Regelson & Holland* 1958) have been inhibited Our present interest in these substances was aroused by the finding that a polyphosphate seemed to exercise an effect on the growth of cells (*Saxen & Penttinen* 1962) which was very similar to that previously described as due to fresh individual human sera (*Penttinen & Saxen* 1956 and 1959, *Saxen & Penttinen* 1961) The cells did not grow in the more common, loose mosaic structures, but in dense clumps only weakly attached to the glass surface Furthermore, the same polyphosphate has been found to agglutinate red blood cells and to inhibit influenza virus in eggs (*Penttinen* 1956) and herpes virus in cell culture (*Vaheri & Penttinen* 1962) Special interest is attached to the observation that heparin, a polyanion of animal origin, seemed to share the biological effect of the above polyphosphate on the growth of cells (*Saxen & Penttinen* 1962) and on herpes simplex virus (*Nahmias & Kibrick* 1963, *Vaheri & Cantell* 1963)

The current comparative studies were embarked upon in an endeavour to determine possible common features in the biological effects of polyanions on herpes simplex virus blood coagulation, red blood cells and the growth behaviour of cells on a glass surface To this end, there were available a number of polyanions developed, originally as enzyme inhibitors (*Diczfalusy et al* 1953), in the laboratories of the Leo Company pharmaceutical manufacturers, Halsingborg, Sweden[1]

The studies, very interesting in principle with respect to the similarity in the effect of fresh human sera and polyanions on cell attache-

Supported by a research grant from the Sigrid Juselius Foundation

[1] We are greatly indebted to Drs *B Hogberg* and *H Fex* of the Leo Company

ment and growth behaviour on a glass surface, are reported separately (Nordling et al 1964 b)

MATERIAL AND METHODS

Polyanions

The characteristic properties of the polyanions investigated have been compiled in Table 1[1]

* The figures given after the primary and secondary phosphates represent their

†

Leo 114 C)
§ From dextran molecular weight about 60 000
° Hydrolysis entails that the primary phosphates in Leo 116 C are columned off The degree of polymerization is accordingly unchanged

The polyanions 137 B 161 A 160 A and 164 A are easily dissolved in water the other polyanions are soluble in mild alkali

Heparin A commercial preparation of sodium heparin (Medica Helsinki) containing about 100 IU per mg was used

Determination of heparin like antithrombin activity A quantity of 0 2 ml citrated bovine plasma (9 parts to 1 part of anticoagulant) and 0 2 ml of the test substance diluted with buffer (Waaler s dilution fluid II) were incubated for 3 minutes at room temperature The mixture was clotted with 0 2 ml of thrombin (Topostasine® La Roche) solution which gave a clotting time of 10–14 seconds in this system with buffer only

A reference curve was prepared with the aid of known concentrations of heparin (2 5 µg per ml of plasma) For each test substance there were selected two dilutions which gave clotting times fitting the reference curve From the reference curve the quantity of the test substance was calculated which in this test system had the same

tities of the test substances op
ere added to bovine plasma These
agent hexadimethrine bromide
(Polybrene®) by Godal s (1961) thrombin system The results are expressed as quantities of the test substances neutralized by 1 µg of Polybrene®

nt lactalbumin hydrolysate served as
each dish the final volume was 1 ml
in a humified 5 per cent CO_2 atmos
s the medium was removed and the
monolayers were supplemented by 2 ml of 1 5 per cent carboxymethyl cellulose overlay medium with no neutral red After 3 4 days of incubation at 37° C the plaques could be
instances only
final concentra
The quantit
... per cent of the number of

[1] Data obtained from the Leo Company

TABLE 1

Polymers, Characteristic Anionic and Cationic Groups Approximate Molecular Weight and Hyaluronidase Inhibition

Leo no	Polymers	Characteristic anionic and cationic groups*	Molec weight	Hyase inhibition†
131 A	Polyvinylalcohol with acid esters of phtalic acid	carboxylates, arom + aliph, ½/unit	20000	0 1
137 B	Polyphloroglucinol phosphate	phosphates, sec and possibly some prim	polydisperse	0 1
127 A	Dextran with acid esters of phtalic acid	carboxylates, arom, 2/unit	60000§	0 2
175 A	Copolymer of styrol and maleic acid, 1 1	carboxylates aliph	80000	0 2
116 F	Polystilbol phosphate of high molec weight	phosphates prim (10) and sec (70–80)	> 14000	0 6
116 C	Polystilbol phosphate of medium molec weight	phosphates, prim (25) and sec (60–70)	14000	0 6
153 A	Formaldehyde polymers of p oxybenzoic acid	carboxylates, arom	? > 5000	0 7
114 C	Polyestradiol phosphate	phosphates prim (30) and sec (60)	25000	1 0
140 A	Poly bisphenol A phosphate	phosphates prim (20) and sec (60–70)	?	1 0
139 C	Polystilbol phosphate hydrolyzed	phosphates sec and no prim	14000°	1 3
155 A	Formaldehyde polymers of [illegible]	[illegible]		

116 C	Polystilbol phosphate of low molec weight	phosphates (70 80)		
101 A	Polyphloretin phosphate	phosphates prim (70) and sec (?)	15000	15
154 A	Formaldehyde polymer of anthranilic acid	carboxylates arom, basic groups possibly	? > 5000	2
152 A	Formaldehyde polymer of salicylic acid	carboxylates arom	< 5000	2
142 B	Polyquercetin phosphate	phosphates prim (?) and sec (?)	?	3
130 A	Pectinic acid with acid esters of phtalic acid	carboxylates, arom + aliph 2/unit	> 50000	5
129 A	Dextran with acid esters of phtalic acid	carboxylates, arom, 1/unit	60000§	20
176 A	Copolymer of methylmethacrylate and maleic acid, 2 1	carboxylates aliph	> 10000	100
132 A	Rutin estered with 5 6 acid phtalic acid groups	carboxylates, arom + aliph, 5-6 units	1500	175
128 A	Dextran with acid esters of phtalic acid	carboxylates arom, ½/unit	60000§	500
151 A	Tetra Na salt of stilbol disphosphate	phosphates, prim (100)	516	800
164 A	Polyrutin phosphate of low molecular weight	phosphates prim, (70) and sec (?)	?	1000
160 A	Polyphloretin phosphate with also basic groups	polyampholyte	> 15000	—

* † § ° See Material and Methods

plaques from the average control figures was calculated as follows The average percentage of the reduction of the plaque number was arrived at for each concentration of the polymers, and the 50 per cent reducing value was interpolated linearly from these percentages

Effect on chicken red cells The substances were titrated on plastic plates like haemagglutinating agents, using 0 5 per cent chicken red cells and an incubation time of 2 hours at room temperature Phosphate buffered saline without Ca^{++} and Mg^{++} served as the diluent for the substances and the red cells both of which were added in a volume of 0 25 ml The results in Table 2 are given as the minimal 'haemagglutinating' concentration of the polymers diluted in steps

The clumping test HeLa cells maintained in 30 per cent inactivated human serum pool in Hank's salt solution were used Trypsinization was effected by Difco 1/250 trypsin, with a calculated final concentration of 0 16 per cent of trypsin in Hank's salt solution The period of incubation was 30 minutes at 37° C The inoculum size was approximately 30 000 cells The test tubes (16 × 120 mm) were washed in mild alkali and sterilized in a heat oven (*Nordling et al* 1964 a) The polymers were diluted in the culture medium which consisted of 30 per cent aged pooled serum in Hank's salt solution The growth behaviour was recorded after 24 hours' stationary incubation, *strong clumping* being recorded when cells were not attached to the glass, but to each other as freely moving large clumps and *clear clumping* indicating attachment of the cells to the glass surface as dense clumps The dilution of polyanion sufficient to cause this degree of clumping is given in Table 2 *No clumping* was recorded for cells growing in a loose structure and forming mosaic like sheets

RESULTS

The results have been compiled in Table 2 which gives the antithrombin effect, the effect of Polybrene® titration, metachromatic activity, *the rate of herpes virus inhibition, the haemagglutinating* activity on chicken red cells (no agglutinating activity was observed when guinea-pig or human red cells were used), and the clumping effect of the polyanions on HeLa cells grown on a glass surface From these findings, along with the figures in Table 1, it is evident that the hyaluronidase inhibition of the polyanions shows a general pattern which is similar to that of the effects observed in the present investigation The deviations from the general pattern are few By measurement of the effect of a polyanion in one biological function it is almost possible to predict the actions in other systems

Chemically and physically, the present polyanions constitute a very heterogeneous group (see Table 1) They include various types of anionic macromolecules with aromatic and aliphatic carboxylates, primary and secondary phosphates or sulfonates The substances also include certain groups of polymers which permit a closer comparison between the physicochemical structure (Table 1) and the bio-activities (Table 2)

(a) The molecular size of the stilbol phosphates (151 A, 116 G, 116 C and 116 F) appears to increase in parallel with the intensity in their different biological actions

(b) The amount of acid esters of phtalic acid in dextran (128 A, 129 A and 127 A) is in close correlation with the degree of biological effectiveness of these carboxylate polymers

(c) When the primary phosphates in polystilbol phosphate (116 C)

are hydrolyzed off (139 G), there seems to a corresponding drop in the bio activities

(d) The addition of basic groups to polyphloretin phosphate (101 A) appears to impair the activity of the polymer (160 A)

TABLF 2

Comparison of Biological Activities of Heparin and Certain Synthetic Polyanions The Polyanions Are Given According to their Antihyaluronidase Activity

Polyanion	Anti thrombin effect*	µg neutra lized by 1 µg Poly brene®	Metachro matic activity	Inhibition of herpes virus†	Haemag glutinating activity‡	µg ml causing clear clumping of Hela cells
Heparin	1	1 1	+°	0 1	No effect	3 6
131 A	3 4	1	+	0 2	0 5	1 5
137 B	2 2	0 4	+	0 2	1	6
1?7 A	4 6	2	+	0 2	1	6
175 A	2 2	1	+	2 4	1	3
116 F	20 25	0 5	+?	0 6	0 5	6
116 C	40–50	3	—?	1 4	1	6
153 A	30–40	3	+	0 3	2 5	6
114 C	25 30	5	—	1 1	1	25
140 A	90–90	8	—?	1 5	10	12
139 C	80–300	100		2.3	2 5	12
155 A	6–8	2	+	1 1	5	25
116 G	45–45	25	+?	2 1	2 5	12
101 A	30–50	5	+	1 3	25	250
154 A	3 5	25	?	1 9	10	250
15? A	80–150	17	—	1 0	1000	50
142 B	60–110	10	?	1 8	5	50
130 A	3 3	0 3		2 4	50	25
129 A	6–6	1 2	—	1 0	2500	6
176 A	3 3	0 5	—	18	2500	12
132 A	300–300	0 3	—	40	No effect	No effect
128 A	25–30	2 5	—	80	No effect	No effect
151 A	No effect	—	—	No effect	No effect	No effect
164 A	50–200	12	—	10	No effect	No effect
160 A	300–330	650	—	No effect	No effect	100
						No effect

The quantity in µg per ml with the same antithrombin activity as 1 µg heparin per ml

† The quantity in µg per ml reducing the number of plaques by 50 per cent

‡ Minimum quantity in µg per ml causing haemagglutination of chicken red cells

° Macroscopic metachromatic staining of toluidine blue with 100 µg per ml of polyanion.

DISCUSSION

The available evidence presented above seems to indicate that this biological activity of the polyanions may be correlated with the net amount of anionic groups (electronegativity) and the molecular size, to which the anticoagulant activity of heparin (*Engelberg* 1963) is said to be related

The so-called heparinoids have thus far been sulphated esters of large molecular compounds mostly of polysaccharides polyuronides and mucopolysaccharides but also of synthetic macromolecules (*Pulver* 1961) Studies of antithrombin activity and neutralization by Poly-

brene® appear to indicate that the active polyanions investigated and not only sulphated esters, act like heparin, and can thus be termed heparinoids

The metachromatic reaction, which depends primarily on the formation of bonds between the basic amino groups of the dye and the anionic groups of heparin and heparinoids, is said to be reasonably specific in the presence of NaCl *in vitro* (*Jaques & Bell* 1959) The experiments with toluidine blue further corroborate the heparinoid nature of the present macromolecules

Nevertheless, marked differences exist between the bio activities of the natural polyanion, heparin and the present synthetic polyanions The synthetic polyanions had anticoagulant functions in the thrombin fibrinogen system Thus they did not need a cofactor, which is a well established pre-requisite for the antithrombin activity of heparin Secondly, in spite of its potent actions in many biological systems heparin did not agglutinate chicken red cells Thirdly, it seems that the effect of heparin on cell attachment and growth behaviour is different from that caused by either fresh human sera or the present synthetic polyanions (*Nordling et al* 1964 b) Furthermore, investigation on the mechanism of the antiviral action of heparin and other polyanions (*Vaheri* 1964) has emphasized other, presumably associated divergences All polyanions inhibited the early interaction of herpes virus and the cell, but the relation between reversible ("dissociable") and irreversible ("virucidal") action on the virus, in this inhibition showed wide variations for the different polyanions Thus heparin had only reversible action on the virus, many synthetic polyanions were primarily virucidal and certain heparinoids appeared to hold a mid-position The degree of virucidal action of the polyanions seemed to correlate with their ability to agglutinate red cells (see also Table 2) and their toxicity to cell cultures

There is evidence that both the primary attachment of viruses to cells (*Philipson* 1963) and of cells to surface (*Nordling et al* 1964 a) might be electrostatic It is conceivable that heparin and other polyanions by increasing the net negative charge of viruses and cells (*Allison & Valentine* 1960), might exercise comparable inhibitory effects on the attachment of herpes virus to cells and HeLa cells to a glass surface It is also possible that the effects may be comparable with the action of heparin and these polyanions on blood platelets However, since heparin and the present polyanions are strongly inhibitory on herpes virus in saline medium (*Vaheri* 1964) but apparently do not prevent cell attachement without serum (cofactor or carrier) (*Nordling et al* 1964 b), it is likely that the mechanisms differ

The present investigation emphasizes the broad bio-activities of polyanionic macromolecules The diverse actions of heparin, and other polyanions can be supposed to derive from their general reactivity with basic sites in many proteins and other substances (*Jaques & Bell*

1959) Accordingly, there is no justification of farreaching generalizations on the different biological actions of polyanions

SUMMARY

Various polyanions (with aromatic or aliphatic carboxylates, primary or secondary phosphates or sulphonates) have 1) marked inhibitory effect on herpes virus, 2) potent heparin-like anticoagulant action, also without cofactor, 3) metachromatic activity, 4) agglutinating action on chicken—but not human or guinea pig—red cells, 5) growth controlling (clumping) effect in the presence of serum on the growth of HeLa cells on a glass surface

The bio-activities are correlated with the amount of anionic groups and the molecular size of the polymers The biological actions of heparin and synthetic polyanions have been compared

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CHOLERA FILTRATE AND VASOPRESSIN

Antagonistic Effect on the Isolated Frog Skin

By

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Received 8 iv 64

During the past 15 years strong evidence has accumulated that the classical concept of Asiatic cholera is incorrect The denuadation of the intestinal epithelium claimed to be caused by the vibrio was not found on repeated investigations A new theory postulated an inhibition of the active transport of sodium as the cause of the diarrhoea Much of this evidence is collected in studies conducted by U S Naval Medical Research Units and it is reviewed by *Phillips* (1963) He also presents evidence of a sodium pump inhibitor in stools of cholera patients

A similar inhibitor was found by *Fuhrman* in a commercial cholera filtrate (*Fuhrman & Fuhrman* 1960 *Fuhrman Fuhrman & Burrows* 1962) The preparation used is produced by Philips Duphar as a source of neuraminidase which is an enzyme utilized in influenza diagnosis When the Cholera Filtrate was brought into contact with the anatomical outside of the isolated frog skin the active transport of sodium was strongly inhibited No inhibition was found when the filtrate was applied to the inside of the skin

Several but not all batches of this preparation were inhibitory Attempts to produce active filtrates from different substrates and with different strains of V *cholerae* were almost fruitless and only one preparation showed slight inhibition Endotoxin produced by the method of Westphal proved inactive as well as several other toxin preparations

The inhibiting factor was dialysable and a molecular weight of a few hundred was suggested It was negatively charged at neutral pH and was relatively stable to heating acid and alkali

MATERIAL AND METHODS

Experiments were performed mostly with the skin from the European [illegible]

The study upon which this paper is based received financial support from the *World Health Organization*

was equipped with a meter for measuring the potential difference between the two compartments and with a short circuiting device composed of a 108 volt anode battery, a potential divider and a micro-ammeter This makes it possible to short circuit the frog skin by the application of an external electromotive force, and then to measure the current produced by the skin under these conditions The current measured in this way is the so called 'short circuit current' *Ussing & Zerahn* showed that the short circuit current is equal to the net flux of sodium ions when current and flux are expressed in the same units

During experiments the potential divider was constantly adjusted to maintain the potential difference across the skin at zero For measuring the potential under normal conditions the anode battery was only momentarily disconnected

Cholera Filtrate was obtained from N V Philips Duphar, Amsterdam[1] It was received freeze dried in ampoules, and each ampoule was dissolved in 10 ml of 'NaCl free frog Ringer solution' The filtrate contains no bacteriostatic

Vasopressin was obtained from A/S Alfred Benzon Copenhagen under the name 'Insipidin' It was claimed to contain 20 International Units (I U) per ml of vasopressor substances

The copper solution was a solution of $CuSO_4$, 5 H_2O The concentration was 625 mg per litre 0 1 ml of this solution in 25 ml gives a final concentration of 10^{-5}M

The anti-cholera serum was a pool from ten rabbits immunized with the International Reference Preparation of Cholera antigen (Inaba)

The frog-Ringer solution was composed as follows

$NaHCO_3$	0 20 g
KCl	0 20 g
NaCl	6 50 g
$CaCl_2$ (solution 24 % w/v)	0 41 ml
Redistilled water to	1000 ml

In the 'NaCl free frog Ringer solution' the NaCl was omitted

RESULTS

Fig 1, a–d, shows the effect of Cholera Filtrate on the short-circuit current An inactive filtrate provoked only a slight and reversible rise in current (1 a), whereas the active filtrate caused a rapid, pronounced decrease after an initial rise (1 b) This takes place when the filtrate is added to the outside solution When the same dose is added to the inside solution only a very slight and transitory fall in current follows (1 c), but contrary to *Fuhrman*, the addition of four times as much to the inside, caused an immediate, precipitous decrease No initial rise was observed in this case (1 d) Regarding the addition of vasopressin, see below

As the decrease after external application was irreversible, thus making a proper control experiment on the same skin impossible, we tried to find another control method A useful tool was found in the neurohypophyseal hormones It is well-known (*Fuhrman & Ussing* 1961), that these hormones have a decidedly increasing effect on the short-circuit current when they are added to the inside solution Depending on the doses applied this effect was more or less abolished by Cholera Filtrate

[1] We are grateful to Dr *Hertzberger* Philips Duphar for supplying samples of several batches of Cholera Filtrate

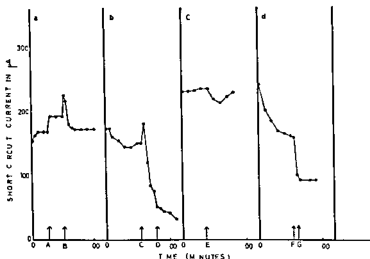


Fig 1

The effect of Cholera Filtrate

a	A 10 ml inactive Cholera Filtrate to outside
	B Outside solution changed
b	C. 0.5 ml active Cholera Filtrate to outside
	D 0.2 I.U. vasopressin to inside
c	E 0.5 ml active Cholera Filtrate to inside
d	F 2.0 ml active Cholera Filtrate to inside
	G 0.2 I.U. vasopressin to inside

The effect of 0.2 I.U. vasopressin on the normal untreated frog skin, is demonstrated in Fig 2 whereas application of vasopressin after active Cholera Filtrate is shown in Fig 1 b A close correlation has been observed between the decrease in current and the degree of inhibition of vasopressin effect produced by different doses of the same filtrate This may be used for quantitative estimation of Cholera Filtrates

Sometimes the frog skin will never or only very slowly, reach a stable short circuit current In such cases a stabilization may frequently be obtained by the addition of Cu (*Ussing* 1955) It is demonstrated in Fig 3 that the skin is still sensitive to Cholera Filtrate after this treatment

In Fig 3 the potential difference across the skin when the external electromotive force is disconnected is also recorded As in this experiment we always found the effect of Cholera Filtrate on the potential difference very slight This disagrees with the findings of *Fuhrman*

Even when the skin is not stabilized it is possible to demonstrate the activity of Cholera Filtrate The effect of vasopressin on such an unstable skin preparation is shown in Fig 4 a and in Fig 4 b the effect of almost simultaneous application of vasopressin and Cholera Filtrate is presented In the latter case the effect of vasopressin is almost blocked

was equipped with a meter for measuring the potential difference between the two compartments and with a short circuiting device composed of a 108 volt anode battery, a potential divider and a micro ammeter This makes it possible to short circuit the frog skin by the application of an external electromotive force, and then to measure the current produced by the skin under these conditions The current measured in this way is the so called 'short circuit current' *Ussing & Zerahn* showed that the short circuit current is equal to the net flux of sodium ions when current and flux are expressed in the same units

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NaCl	6 50 g
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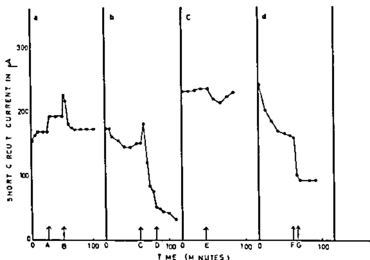


Fig 1

The effect of Cholera Filtrate

a	A 10 ml inactive Cholera Filtrate to outside
	B Outside solution changed
b	C 0 5 ml active Cholera Filtrate to outside
	D 0 2 I U vasopressin to inside
c	E 0 5 ml active Cholera Filtrate to inside
d	F 2 0 ml active Cholera Filtrate to inside
	G 0 2 I U vasopressin to inside

The effect of 0 2 I U vasopressin on the normal, untreated frog-skin, is demonstrated in Fig 2, whereas application of vasopressin after active Cholera Filtrate is shown in Fig 1 b A close correlation has been observed between the decrease in current and the degree of inhibition of vasopressin effect produced by different doses of the same filtrate This may be used for quantitative estimation of Cholera Filtrates

Sometimes the frog-skin will never, or only very slowly, reach a stable short circuit current In such cases a stabilization may frequently be obtained by the addition of Cu (*Ussing* 1955) It is demonstrated in Fig 3 that the skin is still sensitive to Cholera Filtrate after this treatment

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The copper solution was a solution of $CuSO_4 \cdot 5\,H_2O$. The concentration was 625 mg per litre. 0.1 ml of this solution in 25 ml gives a final concentration of 10^{-5} M.

The anti cholera serum was a pool from ten rabbits immunized with the International Reference Preparation of Cholera antigen (Inaba).

The frog Ringer solution was composed as follows:

$NaHCO_3$	0.20 g
KCl	0.20 g
NaCl	6.50 g
$CaCl_2$ (solution 24 % w/v)	0.41 ml
Redistilled water to	1000 ml

In the NaCl free frog Ringer solution the NaCl was omitted.

RESULTS

Fig. 1, a–d, shows the effect of Cholera Filtrate on the short circuit current. An inactive filtrate provoked only a slight and reversible rise in current (1 a), whereas the active filtrate caused a rapid, pronounced decrease after an initial rise (1 b). This takes place when the filtrate is added to the outside solution. When the same dose is added to the inside solution only a very slight and transitory fall in current follows (1 c), but contrary to *Fuhrman*, the addition of four times as much to the inside, caused an immediate, precipitous decrease. No initial rise was observed in this case (1 d). Regarding the addition of vasopressin, see below.

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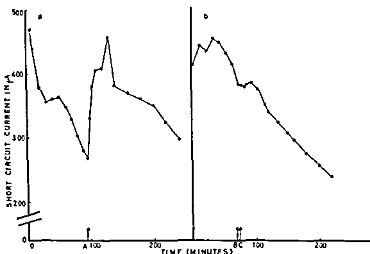


Fig 4

The effect of vasopressin and Cholera Filtrate on unstable skin

a A 0 2 I U vasopressin to inside
b B 1 I U vasopressin to inside
C 0 5 ml active Cholera Filtrate to outside

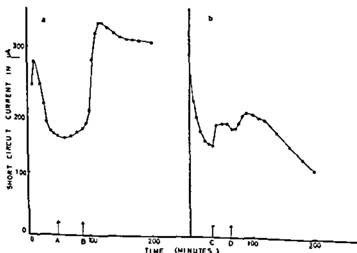


Fig 5

The effect of Anti Cholera Serum

a A 0 1 ml Anti Cholera Serum to outside
B 0 2 I U vasopressin to inside
b C 0 5 ml active Cholera Filtrate incubated for 19 hours at 4° C with 0 1 ml Anti Cholera Serum to outside
D 0 2 I U vasopressin to inside

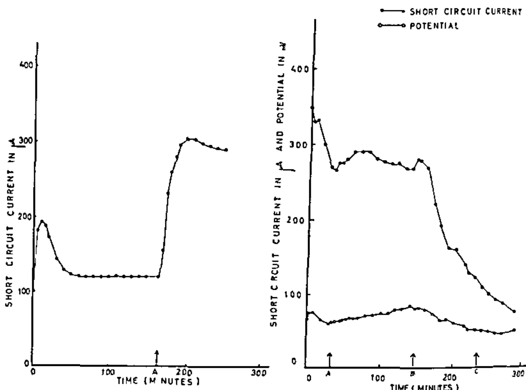


Fig 2

Fig 3

Fig 2

The effect of vasopressin on the frog skin

A 0 2 I U vasopressin to inside

Fig 3

The effect of Cholera Filtrate on the Cu^{++}-treated skin

A 0 1 ml $CuSO_4$ solution to outside

B 0 5 ml active Cholera Filtrate to ouside

C 0 1 ml Anti Cholera Serum to outside

It was also found of interest to see whether an anti cholera serum had any effect on the response In Fig 5 a the addition of 0 1 ml of serum had no effect on the short-circuit current or on the ability of the skin to react to vasopressin In Fig 3 the same dose could not reverse the effect of Cholera Filtrate added previously In Fig 5 b, however, the skin was treated with 0 5 ml of Cholera Filtrate, previously incubated with 0 1 ml of serum The inhibitory effect on the current was effected whereas the vasopressin response was only partially inhibited

DISCUSSION

The theory that the effect of the vibrios in Asiatic cholera is to inhibit the active transport of sodium from the lumen of the intestine to the blood, looked very promising to us This paper describes experiments confirming earlier observations made by *Fuhrman* about the

The possibility is mentioned for utilizing this antagonism for a quantitative estimate of the frog skin paralyzing substance

It is demonstrated that incubation of the Cholera Filtrate with an anti cholera serum prior to addition to the frog skin inhibits the paralysis

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presence of a substance in 'Cholera Filtrate, Philips-Duphar' inhibiting the short-circuit current in isolated frog-skin

It is confirmed that when the Cholera Filtrate is added to the outside of the skin, a rise in current will occur initially, but after 15 20 minutes the current will start to decrease The steepness and size of this decrease seem to be dependent on the dose of Cholera Filtrate *Fuhrman* found that Cholera Filtrate had no such effect when added to the anatomical inside of the frog skin We, however, have shown that an inhibitory effect is also present in that case, but with a somewhat lower sensitivity The initial increase seen after external application did not occur after *internal* application This may be explained by the presence of the 'sodium-pump' near the inside surface of the skin If the response is the result of a competition between an unspecific stimulation of the skin (*e g* a nutritional effect) and the inhibition of the 'pump', then it will take some time for the inhibitor to reach the point of attack from the outside During this time the stimulatory effect will dominate The difference in sensitivity of the two surfaces is not so easily explained

Because of the irreversibility of the Cholera Filtrate response, and because unspecific decreases or altogether unstable skins were sometimes encountered, the vasopressin effect was investigated This 'check' has been very useful, although it will not distinguish between an unspecific metabolic inhibition and a specific inhibition of the 'sodium pump' The antagonistic effect of vasopressin may be used for quantitative estimation of the inhibitor but due to the slowness of the reaction such an estimation will be rather laborious

The reaction between the inhibitory factor and anti-cholera serum has not been very thoroughly investigated because of shortage of active Cholera Filtrate In the experiment described in Fig 5 b, perhaps the vasopressin was added too early A modification of the response is, however quite obvious It should be mentioned that the serum used was prepared by means of antigen from the Inaba type of *V cholerae*, whereas the strain 4Z, used for the production of the Cholera Filtrate, belongs to the Ogawa serotype

We have not investigated the nature of the inhibitor very closely this being due to shortage of material also We have, however, been able to confirm that it is dialysable and, at least to some degree, heat-stable

SUMMARY

Fuhrman's discovery is confirmed that 'Cholera Filtrate, Philips-Duphar' may contain a heat-stable, dialysable frog-skin paralyzing substance

An antagonistic effect between cholera filtrate and vasopressin on the frog-skin is demonstrated It is found also to be effective on the Cu^{++}-treated skin

inactivation than syphilitic reactions (1) This constituted an additional reason for recommending heat inactivation However by a thorough study of this problem it was demonstrated that the heat lability of the reaction was by no means a safe criterion for distinguishing non syphilitic from syphilitic reactions (7)

The introduction of the purified phospholipid antigen cardiolipin (9) gave a much better defined antigen for the Wassermann complement fixation test but did not alter the significance or the side effects of preliminary heat inactivaion of serum (2 12)

According to *Schmidt* (12) heat inactivation usually results in a reduction in the titre of both syphilitic and non syphilitic antisera the syphilitic generally being the more heat stable Inactivation of native complement would be expected to reduce haemolysis thus leading to higher titres being recorded Since the reverse is usually the case changes tending to enhance haemolysis seem to be the predominant result of heat inactivation

The reduction in titre registered after heat inactivation might be due either to characteristics of the antilipoidal immune globulins which themselves may be susceptible to thermal influences or to other serum components whose influence on the complement fixation test changes with heat inactivation

The purpose of the present stury is to investigate the influence of various serum components on the outcome of the Wassermann complement fixation test in reactive syphilitic sera as well as the variations in this influence after heat inactivation

MATERIAL AND METHODS

Serum and Cerebrospinal Flui l (CSF)

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Wassermann Complement Fixation Test with Card ol p n Antigen

The standard procedure in this laboratory is Morch s modification of the or ginal Wassermann test using cardiolipin ant gen This technique has been descril ed in deta l by *Schm dt* (10)

... aries from 0 320 to 0 360 ml the ... ure of buffered sal ne conta ning the measured amounts of antigen and guinea p g c mplement being constant (0 300 ml)

The complement fixation process is allowed two hours at 4° C and 30 minutes at

Acta path et microbiol scandinav 62 356-366, 1964

The Treponematoses Department, Statens Seruminstitut, Copenhagen Denmark

STUDIES ON ANTILIPOIDAL IMMUNE GLOBULINS

1 *Influence of Normal Components of Human Serum and Cerebrospinal Fluid on the Outcome of the Complement Fixation Test with Cardiolipin Antigen, and Variation in this Influence Following Heat Inactivation*

By

ERIK HOLST

Received 8 ix 64

Most serological examinations in complement fixation tests are preceded by thermal conditioning of the serum, called heat inactivation, usually consisting of 30 minutes' incubation of serum samples in open tubes on a waterbath maintained at a constant temperature of 56° C This procedure was originally introduced in order to inactivate native human complement which might otherwise supplement to varying extents the well-defined amount of guinea-pig complement added in the complement fixation test

Heat inactivation results in irreversible destruction of the thermo labile complement factors C'1 and C'2 The other complement factors C'3a, C'3b and C'4 are more resistant to heat but also suffer partial or total loss of activity during the usual heat inactivation procedure (5)

Although the wish to inactivate human complement was the theoretical background for using heat inactivation of the serum as a standard procedure prior to examination in the Wassermann complement fixation test for detection of antilipoidal immune globulins in syphilis, it soon became obvious that this was far from being its only effect

A number of sera give more conclusive results after heat inactivation This has been ascribed to a destruction of thermolabile native haemo lysins during heat inactivation (3) and is also due to the fact that a number of sera which are slightly anticomplimentary in the unheated state fail to show anticomplementary activity after heat inactivation On the other hand, some sera—especially from cases of paraprotein aemia—may develop a strongly anticomplementary activity after heat inactivation, without showing any anticomplementry activity in the unheated state (8) Some sera show reactivity before but not after heat inactivation (13), and many give higher titres before than after heat inactivation (6) However, with some sera the reverse was the case (3)

Non-syphilitic reactions in the Wassermann complement fixation test with the original crude lipid antigen seemed more susceptible to heat

inactivation than syphilitic reactions (1) This constituted an additional reason for recommending heat inactivation However by a thorough study of this problem, it was demonstrated that the heat lability of the reaction was by no means a safe criterion for distinguishing non syphilitic from syphilitic reactions (7)

The introduction of the purified phospholipid antigen cardiolipin (9) gave a much better defined antigen for the Wassermann complement fixation test but did not alter the significance or the side effects of preliminary heat inactivaion of serum (2 12)

According to *Schmidt* (12), heat inactivation usually results in a reduction in the titre of both syphilitic and non syphilitic antisera, the syphilitic generally being the more heat stable Inactivation of native complement would be expected to reduce haemolysis thus leading to higher titres being recorded Since the reverse is usually the case, changes tending to enhance haemolysis seem to be the predominant result of heat inactivation

The reduction in titre registered after heat inactivation might be due either to characteristics of the antilipoidal immune globulins which themselves may be susceptible to thermal influences or to other serum components whose influence on the complement fixation test changes with heat inactivation

The purpose of the present stury is to investigate the influence of various serum components on the outcome of the Wassermann complement fixation test in reactive syphilitic sera, as well as the variations in this influence after heat inactivation

MATERIAL AND METHODS

Serum and Cerebrospinal Fluid (CSF)

The 3 [illegible]

Wassermann Complement Fixation Test with Cardiolipin Antigen

The standard procedure in this laboratory is Morch s modification of the original Wassermann test using cardiolipin antigen This technique has been described in detail by *Schmidt* (10)

[illegible] complement fixation process is allowed two hours at 4° C and 30 minutes at

37° C before the addition of sheep blood (2 5 per cent suspension in saline) and amboceptor

Haemolysis is read after 1 hour at 37° C and 18 hours at 4° C by comparison with a colour scale prepared each day Titres are expressed in $\log_{10}$ values of the dilutions giving 50 per cent haemolysis calculated according to the *Kärber* method (4)

The guinea pig complement titre is determined every other day against amboceptor and sheep blood The amboceptor titre is kept constant for longer intervals (6 months)

The antigen consists of 0 0175 per cent (w/v) cardiolipin 0 0875 per cent (w/v) egg lecithin (both prepared at the Statens Seruminstitut), and 0 3 per cent (w/v) cholesterol (Pfanstiehl) in dehydrated ethanol

Examination of serum for content of native human complement was performed in the same way as the quantitative Wassermann reaction, except that the addition of guinea pig complement was omitted

EXPERIMENTAL DATA

1 As it can be seen from the description of our method, the quantitative determination of the antilipoidal immune globulins in the Wassermann complement fixation test is accomplished by performing a serial three fold dilution of serum in physiological saline

This means that the titration end-point is determined in the presence of higher concentrations of other scerum components for weak antisera than for antisera with high titres

In order to eliminate this difference the quantitative determination of antilipoidal immune globulins in eight syphilitic sera was performed by dilution of antiserum in unheated and in heat inactivated non-reactive serum pool from healthy human donors insted of in saline

TABLE 1

Quantitative Examination of Eight Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen Comparison Between Serial Dilutions in Saline Unheated and Heat Inactivated Non Syphilitic Serum

Serum no	Diagnosis	Dilution in saline $\log_{10}$ value	Dilution in unheated non reactive serum pool $\log_{10}$ value	Dilution in inactivated non reactive serum pool $\log_{10}$ value
179	S I	1 193	0 239	0 930
193	S I	0 692	0 239	0 334
182	S II	0 692	0 239	0 286
184	S II	1 431	1 026	1 193
192	S II	1 431	0 811	1 145
180	S lat rec	0 907	0 286	0 334
183	S lat rec	0 716	0 239	0 239
189	S lat rec	0 620	0 239	0 239
Mean $\log_{10}$ value		0 960	0 415	0 588
Mean reduction in titre			0 545	0 372
Mean reduction in titre (unheated/inactivated)				0 173
Standard error			0 057	

A comparison of the results with those of the standard procedure with dilution in saline performed simultaneously is shown in Table 1.

It will be seen from Table 1 that dilution in normal human serum pool, both unheated and heat inactivated, reduces the reactivity of syphilitic sera compared with the reactivity registered after dilution in saline. Dilution in unheated serum pool results in a greater reduction in reactivity that dilution in heat inactivated serum pool, a fact which might be explained by the presence of native complement in the former. However, this experiment demonstrates that other serum components besides the native complement play a rôle in reducing the reactivity. Otherwise it would be impossible to explain the reduction caused by the heat inactivated serum pool. Serum components which inhibit complement fixation have been demonstrated earlier (7, 11) and their possible mode of action has been discussed (14).

2. The total amount of serum, undiluted or diluted, in the non reactive serum pool in the first experiments was 0.020–0.060 ml in each tube according to the standard technique described.

In order to bring out more clearly the influence of the components of the non reactive serum pool, the heat inactivated sera examined in our second experiment were diluted serially in saline, after which 0.3 ml of unheated non reactive serum pool was added to each tube before the addition of guinea pig complement and cardiolipin antigen.

The effect of adding 0.3 ml of heat inactivated serum pool and 0.3 ml of saline to each tube was determined in the same manner.

TABLE 2

Quantitative Examination of Eight Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen in the Presence of 0.3 ml of Saline, Unheated or Heat Inactivated Non Syphilitic Serum Added Prior to Complement Fixation. Results Given in $\log_{10}$ Values

Serum no	Diagnosis	Addition to each tube of 0.3 ml of		
		Saline	Unheated non reactive serum pool	Heated non reactive serum pool
179	S I	1.288	0.000	0.573
193	S I	0.740	0.000	0.310
182	S II	0.787	0.000	0.239
184	S II	1.336	0.000	0.763
19[illegible]	S II	1.455	0.000	0.763
180	S lat rec	0.811	0.000	0.239
183	S lat rec	0.763	0.000	0.191
189	S lat rec	0.620	0.000	0.239
Mean $\log_{10}$ value		0.975	0.000	0.415
Mean reduction in titre			(0.975)	0.560
Mean reduction in titre (unheated inactivated)				(—0.415)
Standard error			0.057	

Table 2 shows the result of these experiments, all of which were performed on the same day

It will be seen from Table 2 as compared with Table 1 that the addition of 0 3 ml of saline to the volume in which the complement fixation takes place (0 3 ml) did not influence the result Therefore the reduction in titre registered after the addition of 0 3 ml of non-reactive serum pool must be due, not to changes in the reaction volume, but to normal human serum components

As might be expected, the reduction in titre is more pronounced than in the first two experiments, the influence of unheated serum pool again exceeding that of heat inactivated serum pool

3 In order to characterize the serum factors responsible for the decrease in reactivity registered on titration in heat inactivated human non-reactive serum pool, a study of the influence of various serum proteins on the outcome of the complement fixation test was performed

The various serum proteins were salted out by dialysis against solutions of ammonium sulphate of varying concentrations The protein which was precipitated with 40 per cent saturation was designated gamma-globulin, that precipitated with 50 per cent saturation alpha and beta-globulin, and that precipitated with 68 per cent saturation albumin

The precipitates were redissolved in saline and dialysed against buffered saline Then a number of syphilitic sera were titrated in each of the three protein solutions as well as in the original non-reactive serum pool from which the serum protein fractions were derived

After dialysis, the heat inactivated serum protein fractions used in this experiment showed no anticomplementary activity in the doses used, *i e* 0 060 ml at most

TABLE 3

Quantitative Examination of Five Heat Inactivated Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen Comparison between Serial Dilutions in Saline Whole Non Syphilitic Serum and Three Heat Inactivated Solutions of Fractionated Non Syphilitic Serum Proteins Results given in $\log_{10}$ Values

Serum no	Diagnosis	Diluted in saline	Diluted in heat inactivated non reactive			
			Whole serum	γ glob	α or β glob	Albumin
241	S I	1 002	0 525	0 716	0 811	0 716
251	S I II	1 431	1 026	1 097	1 288	1 241
243	S II	1 241	0 978	1 097	1 312	1 145
276	S II	1 861	1 169	1 312	1 527	1 575
261	S lat tard	1 193	1 121	1 217	1 241	1 193
Mean $\log_{10}$ value		1 346	0 964	1 088	1 236	1 174
Mean reduction in titre			0 382	0 258	0 110	0 172
Standard error				0 073		

An attempt to add excessive amounts of the individual serum protein fractions to each tube in the titration series was unsuccessful due to the anticomplementary action in high doses of these fractions

It will be seen from Table 3 that each of the protein fractions is able to reduce the titre of syphilitic sera under the conditions of these experiments The variations in their reducing capacity are not significant, although it would appear that the serum proteins precipitated with 40 per cent saturated ammonium sulphate contain most of the reducing capacity demonstrated in whole serum Inhibitors have been demonstrated previously in the albumin fraction precipitated with ammonium sulphate (7) and in the albumin + alpha 1 fraction of Tiselius electrophoresis (11)

4 Since cerebrospinal fluid does not contain complement, heat inactivation is not considered necessary for such samples before complement fixation testing It is also found that the reactivity of cerebrospinal fluid is influenced only slightly by the standard heat inactivation at 50° C for 30 minutes

On this background it has been considered worth while to examine the influence of non reactive cerebrospinal fluid on the reactivity of antilipoidal reactive syphilitic sera

The effect of serial dilution in non reactive CSF instead of in saline is shown in Table 4

TABLE 4

Quantitative Examination of 10 Heat Inactivated Syphilitic Sera in Complement Fixation with Cardiolipin Antigen Comparison between Serial Dilutions in Saline Unheated and Heat Inactivated Non Syphilitic Cerebrospinal Fluid (CSF) Results Given in log_{10} Values

Serum no	Diagnosis	Original dilution in saline	Diluted in	
			Unheated non-reactive CSF pool	Heat inact non reactive CSF pool
303	S I	0 907	0 859	0 954
310	S I	1 431	1 288	1 241
306	S II	1 050	0 859	0 930
307	S II	1 193	0 907	0 859
318	S lat rec	1 241	1 288	1 193
319	S lat rec	0 262	0 310	0 286
299	S lat tard	0 930	0 740	0 811
298	S III	1 622	1 479	1 479
300	S III	2 147	1 622	1 431
301	S III	1 479	1 193	1 288
Mean log_{10} value		1 226	1 055	1 047
Mean reduction in titre (compared to original titre)			0 171	0 179
Mean reduction in titre (unheated inactivated)				0 008
Standard error			0 051	

Table 2 shows the result of these experiments, all of which were performed on the same day

It will be seen from Table 2 as compared with Table 1 that the addition of 0 3 ml of saline to the volume in which the complement fixation takes place (0 3 ml) did not influence the result Therefore the reduction in titre registered after the addition of 0 3 ml of non-reactive serum pool must be due, not to changes in the reaction volume, but to normal human serum components

As might be expected, the reduction in titre is more pronounced than in the first two experiments, the influence of unheated serum pool again exceeding that of heat inactivated serum pool

3 In order to characterize the serum factors responsible for the decrease in reactivity registered on titration in heat inactivated human non-reactive serum pool, a study of the influence of various serum proteins on the outcome of the complement fixation test was performed

The various serum proteins were salted out by dialysis against solutions of ammonium sulphate of varying concentrations The protein which was precipitated with 40 per cent saturation was designated gamma-globulin, that precipitated with 50 per cent saturation alpha and beta-globulin, and that precipitated with 68 per cent saturation albumin

The precipitates were redissolved in saline and dialysed against buffered saline Then a number of syphilitic sera were titrated in each of the three protein solutions as well as in the original non-reactive serum pool from which the serum protein fractions were derived

After dialysis, the heat inactivated serum protein fractions used in this experiment showed no anticomplementary activity in the doses used, *i e* 0 060 ml at most

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Quantitative Examination of Five Heat Inactivated Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen Comparison between Serial Dilutions in Saline Whole Non Syphilitic Serum and Three Heat Inactivated Solutions of Fractionated Non Syphilitic Serum Proteins Results given in log_{10} Values

Serum no	Diagnosis	Diluted in saline	Diluted in heat inactivated non reactive			
			Whole serum	γ glob	α og β glob	Albumin
241	S I	1 002	0 525	0 716	0 811	0 716
251	S I II	1 431	1 026	1 097	1 288	1 241
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276	S II	1 861	1 169	1 312	1 527	1 575
261	S lat tard	1 193	1 121	1 217	1 241	1 193
Mean log_{10} value		1 346	0 964	1 088	1 236	1 174
Mean reduction in titre		–	0 382	0 258	0 110	0 172
Standard error				0 073		

TABLE 6

Examination of Ten Non Syphilitic Sera for Content of Native Complement within 24 Hours after Withdrawal of Blood Sample and after Storage at both 4° C and 20° C

Serum no	% haemolysis within 24 hours					% haemolysis after 3 days at 4° C					% haemolysis after a further 14 days at 20°				
	Control	Serum dilution				Control	Serum dilution				Control	Serum dilution			
		1:1	1:3	1:9	1:27		1:1	1:3	1:9	1:27		1:1	1:3	1:9	1:27
b 2542	100	100	90	5	0	100	90	30	0	0	100	100	50	5	0
b 2544	100	100	60	0	0	100	100	60	0	0	100	100	50	5	0
b 2545	100	100	50	0	0	100	100	20	0	0	100	100	50	0	0
b 2548	100	100	90	10	0	100	100	30	0	0	100	100	50	0	0
b 2549	100	100	80	5	0	100	100	50	0	0	100	100	70	5	0
b 2550	100	100	50	0	0	100	100	20	0	0	100	100	40	0	0
b 2551	100	100	90	5	0	100	100	90	0	0	100	100	90	5	0
b 2552	100	100	80	0	0	100	100	50	0	0	100	100	70	0	0
b 2553	100	100	90	20	0	100	100	60	0	0	100	100	80	5	0
b 2559	100	100	70	0	0	100	100	50	0	0	100	100	30	0	0
Mean % haemolysis	100	100	80	5	0	100	100	50	0	0	100	100	60	5	0
50% Haemolysis titre in [illegible] value			0 643					0 477					0 549		

It will be seen that titration in CSF affects the reactivity measured in the Wasserman complement fixaion test However, there is no appreciable difference in this respect between unheated and heat inactivated CSF

In order to get a clearer picture of the influence or non-reactive CSF pools on the reaction, the experiment was repeated, this time adding 0 3 ml of unheated CSF pool to each tube in one titration row and 0 3 ml of heat inactivated CSF pool to another titration row of the same sera In a control series, 0 3 ml of saline was added to each tube (see Table 5)

TABLE 5

Quantitative Examination of Ten Heat Inactivated Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen in the Presence of 0 3 ml of Saline Unheated or Heat Inactivated Cerebrospinal Fluid (CSF) Added Prior to Complement Fixation Results Given in $\log_{10}$ Values

Serum no	Diagnosis	Addition to each tube of 0 3 ml of		
		Saline	Unheated CSF pool	Heat inactivated CSF pool
303	S I	0 763	0 716	0 668
310	S I	1 336	1 145	0 811
306	S II	0 907	0 954	0 477
307	S II	0 859	0 811	0 477
318	S lat rec	1 575	1 193	0 763
319	S lat rec	0 573	0 310	0 095
299	S lat tard	1 097	0 716	0 239
298	S III	1 527	1 336	1 050
300	S III	1 670	1 336	1 097
301	S III	1 431	1 145	0 954
Mean $\log_{10}$ value		1 174	0 966	0 663
Mean reduction in titre			0 208	0 511
Mean reduction in titre (unheated/inactivated)			0 303	
Standard error				0 051

It was found from this experiment that there is a difference between the effect of unheated and heat inactivated cerebrospinal fluid pool

The reduction in titre registered on addition of 0 3 ml of CSF pool to each tube is most pronounced when heat incativated CSF pool is used This finding is in contrast to what was found with the serum pool in the previous experiments

This experiment shows that human proteins as found in the CSF, may develop during heat inactivation increased ability to reduce the complement fixing activity of a given serum The presence of native complement in serum makes it difficult to ascertain whether the serum proteins behave in the same way

tion upon such sera is therefore unrelated to the inactivation of native complement

TABLE 7

Examination of Ten Non Syphilitic Sera for Content of Native Complement after Storage at 4° C

Serum no	% haemolysis after 3 days at 4 C					% haemolysis after 17 days at 4 C		
	Control	Serum dilution				Control	Serum dilut	
		1/1	1/3	1/9	1/2		1/1	1/3
b 2521	100	90	20	0	0	anticomplementary		
b 2522	100	90	20	0	0	100	40	0
b 2523	100	100	20	0	0	100	30	0
b 2524	100	90	10	0	0	90	0	0
b 2525	100	90	30	0	0	100	50	0
b 2526	100	100	50	0	0	anticomplementary		
b 2527	100	100	40	0	0	anticomplementary		
b 2528	100	90	20	0	0	100	20	0
b 2529	100	100	90	0	0	100	50	0
b 2530	100	100	30	0	0	anticomplementary		
Mean % haemolysis	100	100	30	0	0	100	30	0
50% haemolysis titre in $\log_{10}$ value			0 381					

SUMMARY AND CONCLUSION

1 a Quantitative determination of antilopoidal immune globulins in syphilitic serum was performed by serial dilution in an unheated non-reactive serum pool instead of saline This resulted in an overall reduction in reactivity as determined from the haemolysis curve

1 b Serial dilution in heat inactivated non reactive serum pool of the same syphilitic sera resulted in a reduction in reactivity which was less pronounced than that following serial dilution in unheated serum pool

2 The addition of 0 3 ml of unheated non reactive serum pool to tube in the serial dilution prior to complement fixation resulted in complete depression of reactivity, i e gave full haemolysis in all tubes

3 The addition of 0 3 ml of heat inactivated non reactive serum pool in a similar way resulted in a reduction but not in complete loss of reactivity

4 Serial dilution in heat inactivated solutions of salted out serum protein fractions from a non-reactive human serum pool indicated that the gamma globulin fraction was the most potent in reducing the complement fixing activity of syphilitic antilipoidal immune globulins The albumin fraction also reduced the reactivity, but its influence was less pronounced

5 The activity of native complement in human serum examined in titration experiments with a number of non reactive sera was only

5 The rôle of native human complement has been elucidated by an experiment in which 10 unheated non-reactive sera were subjected to a quantitative complement fixation test without the addition of guinea pig complement The first examination of the sera took place within 24 hours of the withdrawal of the blood

The experiment was repeated three days later, the sera having been stored at 4° C, and again after a further 14 days during which the sera were stored at —20° C The result of these experiments are shown in Table 6

It will be seen from Table 6 that only the undiluted and the three times diluted serum contained sufficient amounts of native complement to produce appreciable haemolysis in the absence of guinea-pig complement The control experiments show that storage (corresponding quite closely to the usual handling of serum received for these experiments) does not influence the complement activity of the unheated sera to any extent

In order to determine the influence of heat inactivation on this native complement activity, the non reactive human serum pool used in this experiment was examined for complement activity before and after heat inactivation

% haemolysis before inactivation					% haemolysis after inactivation				
Control	Serum dilution 1:1	1:3	1:9	1:27	Control	Serum dilution 1:1	1:3	1:9	1:27
100	100	50	0	0	100	0	0	0	0

If storage at 4° C is prolonged for another 14 days, a marked decrease in the complement activity is registered in some sera, while others become anticomplementary (Table 7)

It may be concluded that the native human complement, as normally found in unheated serum, only affects the outcome of the complement fixation test in the first and second tube of the quantitative Wassermann reaction as carried out in this laboratory

In these two tubes the presence of native complement may result in haemolysis, even though the guinea-pig complement added in the usual Wassermann technique is completely fixed by an antigen/antibody complex

The fact that the presence of nattive complecment in unheated serum only affects the first two tubes in the quantitative determination of complement fixing antilipoidal immune globulins means that strong antisera may show complete inhibition of haemolysis in the first two tubes, in spite of the presence of both native and guinea pig complement The titre found in such cases is thus entirely unaffected by the presence of native complement in unheated serum The influence of heat inactiva-

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STUDIES ON ANTILIPOIDAL IMMUNE GLOBULINS

2 *Reactivity in Lepromatous Leprosy with a Lecithin free Cardiolipin Antigen (Cardchol) after Ordinary and Prolonged Heat Inactivation*

By

ERIK HOLST

Received 8 iv 64

Complement fixing antilipoidal immune globulins which react with both the original crude and the more recently purified phospholipoid antigens have been a common finding in patients suffering from leprosy, especially of the malignant lepromatous type (16)

Up to 1949 it was impossible to say with any degree of certainty whether these reactions were due to concomitant treponemal infection

The introduction of tests for the detection of more specific antibodies, *e g* the Treponema pallidum immobilization tests (9) has made it possible to decide with great accuracy which leprosy patients have suffered from a treponemal infection which might explain the presence of antilipoidal immune globulins The literature on this subject has been reviewed by *Fromm et al* (6)

Most investigators have demonstrated that although a number of those leprous sera reacting with lipid antigens also reacted in the TPI test there still remained some sera from patients with lepromatous leprosy which reacted with lipid antigens but showed no reaction in the TPI test

In one study however the investigator was able to conclude that 95 per cent of the antilipoidal reactions could be ascribed to treponemal infection In that study the reactivity in the TPI test was extremely high (35 per cent) (2)

Previous investigations in this laboratory (13 14) have shown that a number of TPI non reactive sera from patients with lepromatous leprosy reacted in a complement fixation test with ordinary cardiolipin antigen containing lecithin (CLC) However, a much higher incidence of reactivity was found with the lecithin free cardiolipin antigen card chol (CC) which is an ethanolic solution containing 0 0175 per cent (w v) cardiolipin and 0 3 per cent (w/v) cholesterol

The incidence of reactivity and the titre of reactive sera were higher before than after heat inactivation at 56° C for 30 minutes with both antigens However, the reactions with the usual cardiolipin antigen containing lecithin seemed more susceptible to heat inactivation than the reactions with cardchol (15)

apparent in undiluted and three fold diluted unheated serum *i e* in the first two tubes

6 It is concluded from these experiments that it has not been possible to demonstrate any serum factor that increases the complement fixing activity *of the antilipoidal antibodies with cardiolipin antigen The total* effect of normal serum factors is a reduction in the reactivity of these antibodies as determined from the haemolysis curve The effect of unheated normal serum is more pronounced than that of heat inactivated normal serum due among other things to the presence in the former of native complement

7 The reduction in reactivity seen in most sera after heat inactivation cannot be explained as a result of changes in the influence of normal serum components On the contrary heat inactivation seems to make normal serum factors less able to reduce the reactivity of syphilitic sera It is probable, therefore that the reduction in reactivity after heat inactivation of syphilitic sera is due to a direct thermal effect on the antilipoidal immune globulins which may be more or less thermolabile

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STUDIES ON ANTILIPOIDAL IMMUNE GLOBULINS

2 *Reactivity in Lepromatous Leprosy with a Lecithin free Cardiolipin Antigen (Cardchol) after Ordinary and Prolonged Heat Inactivation*

By

ERIK HOLST

Received 8 ii 64

Complement fixing antilipoidal immune globulins which react with both the original crude and the more recently purified phospholipoid antigens have been a common finding in patients suffering from leprosy, especially of the malignant lepromatous type (16)

Up to 1949 it was impossible to say with any degree of certainty whether these reactions were due to concomitant treponemal infection

The introduction of tests for the detection of more specific antibodies, *e g* the Treponema pallidum immobilization tests (9) has made it possible to decide with great accuracy which leprosy patients have suffered from a treponemal infection which might explain the presence of antilipoidal immune globulins The literature on this subject has been reviewed by *Fromm et al* (6)

Most investigators have demonstrated that although a number of those leprous sera reacting with lipid antigens also reacted in the TPI test there still remained some sera from patients with lepromatous leprosy which reacted with lipid antigens but showed no reaction in the TPI test

In one study however the investigator was able to conclude that 95 per cent of the antilipoidal reactions could be ascribed to treponemal infection In that study the reactivity in the TPI test was extremely high (35 per cent) (2)

Previous investigations in this laboratory (13, 14) have shown that a number of TPI non reactive sera from patients with lepromatous leprosy reacted in a complement fixation test with ordinary cardiolipin antigen containing lecithin (CLC) However, a much higher incidence of reactivity was found with the lecithin free cardiolipin antigen card chol (CC) which is an ethanolic solution containing 0 0175 per cent (w/v) cardiolipin and 0 3 per cent (w/v) cholesterol

The incidence of reactivity and the titre of reactive sera were higher before than after heat inactivation at 56° C for 30 minutes with both antigens However the reactions with the usual cardiolipin antigen containing lecithin seemed more susceptible to heat inactivation than the reactions with cardchol (15)

The varying results obtained with these two phospholipoid antigens might reflect differences *between the immune globulins with which* each antigen reacts

Experience with cardehol in syphilis serology rather supports such *a hypothesis With cardehol as antigen*, sera from cases of primary syphilis gave titres equalling or even exceeding those obtained with the usual cardiolipin antigen containing lecithin Sera from later stages of syphilis usually gave significantly lower titres with cardehol (8)

The fact that in TPI non-reactive lepromatous leprosy cardehol reacts with more sera and with higher titres than CLC antigen indicates that the complement-fixing antilipoidal immune globulins found in lepromatous leprosy without concomitant treponemal infection differ from those found in syphilis—at least from those found in the later stages of syphilis

The heterogeneity of the antilipoidal immune globulins involved might, among others qualities, manifest itself as a difference in susceptibility to thermal influences

On this background, it was considered of interest to study in more detail the influence *of heat on the reactivity of leprous sera* with the two antigens, *viz* CLC and cardehol

MATERIAL AND METHODS

Serum The present study comprises serum samples from 32 male patients with lepromatous leprosy

The blood samples were taken in Madras[1] After separation of the clot the native serum samples were transported to Copenhagen by air in sealed glass ampoules without any preservative added and in unheated state at ambient temperature

One serum from Osaka and one serum from Coonor both from patients suffering from lepromatous leprosy and both TPI non reactive were used for a supplementary study together with two sera from Copenhagen both from TPI reactive cases of secondary syphilis

Serology The serological examinations were performed in Copenhagen upon arrival of the sera

All the sera were examined for content of treponemal antibody by means of the Treponema pallidum immobilization test (TPI) (9) and the fluorescent treponemal *antibody test (FTA) (3)*

All the specimens were also examined by means of two modifications of the complement fixation test one with CLC antigen and the other with cardehol antigen The technique has been described previously (11 12) The titre is expressed as the $\log_{10}$ value of the dilution which gives 50 per cent haemolysis calculated according to the *Karber* method (5) After examination in the unheated state each sample was divided into three aliquots which were heat inactivated at 56° C for 30 minutes 60 minutes and 120 minutes respectively and then examined in the two complement fixation tests

The four sera in *the supplementary study* were *divided into eight aliquots each* which were heat inactivated at 56° C for 2 5 10 20 30 45 60 and 120 minutes respectively prior to examination in complement fixation tests with CLC and cardehold antigens

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1 Thanks are due to Dr *C W Chacko* Serologist Madras Medical College for his kind assistance in obtaining the sera for this study and for providing the clinical data regarding the patients

RESULTS

A high incidence of anticomplementary activity in sera from cases of lepromatous leprosy has often been reported (1) 24 of the 32 sera (including both TPI reactive and TPI non reactive sera) showed some anticomplementary activity in the unheated state but none showed such activity after heat inactivation

TABLE 1

Serological Examination of 32 Sera from Cases of Lepromatous Leprosy of Various Types and Varying Duration of Infection Results Given in log10 Values

Serum No	Age years	Duration				CLC antigen Heat inactivation at 56°C			Cardchol antigen Heat inactivation at 56°C		
		Leprosy type	Infect years	TPI	FTA 200	30 min	60 min	120 min	30 min	60 min	120 min
1	39	L 1	5/12	—	N	0	0	0	1 026	0 978	0 716
2	24	L 1	2	—	N	0	0	0	0 095	0	0
3	19	L 2	1/12	—	N	0	0	0	0 716	0 334	0
4	25	L 2	3 12	—	N	0	0	0	0	0	0
5	39	L 2	6/12	+	R	1 050	0 835	0 716	0 811	0 740	0 620
6	25	L 2	2	—	N	0	0	0	0	0	0
7	22	L 2	2	—	N	0 835	0	0	2 028	1 551	1 193
8	27	L 2	3	—	N	0 143	0	0	0 286	0	0
9	30	L 2	3	+	N	0	0	0	0 811	0 740	0 501
10	25	L 2	5	—	N	0	0	0	0 095	0	0
11	45	L 2	5	+	R	0	0	0	0 787	0 501	0 239
12	35	L 2	6		N	0	0	0	1 241	1 097	0 573
13	50	L 2	6	+	N	0	0	0	0	0	0
14	50	L 2	10	+	N	0	0	0	0	0	0
15	23	L 3	1/12	—	N	0	0	0	0 811	0 763	0 716
16	25	L 3	6/12	—	N	0	0	0	0 191	0	0
17	43	L 3	6/12		N	0	0	0	0 286	0	0
18	33	L 3	1	—	N	0	0	0	0 620	0 334	0
19	24	L 3	1	—	N	0	0	0	0	0	0
20	35	L 3	16 12		N	1 145	0	0	1 431	1 193	0 978
21	35	L 3	3	—	N	0	0	0	1 002	0 620	0 429
22	28	L 3	5	—	N	0	0	0	0 549	0 191	0
23	18	L 3	8		N	0	0	0	0	0	0
24	42	L 3	9	+	R	1 026	0 620	0 334	0 740	0 620	0 620
25	35	L 3	9	—	N	0	0	0	0 716	0 596	0 239
26	25	L 3	10	—	N	0	0	0	0	0	0
27	46	L 3	12		N	0	0	0	0	0	0
28	16	L 3	10–16	—	N	0	0	0	2 147	2 098	1 765
29	77	L 3	20	—	N	0	0	0	0	0	0
30	32	L 3	2?		N	0	0	0	0	0	0
31	40	L ?	1 12	+	N	0	0	0	0	0	0
32	3?	L ?	9	+	R	1 050	0	0	0 835	0 501	0

CLC — ordinary lecithin containing cardiolipin antigen

The results of serological examination of the heat inactivated sera are given in Table 1 16 out of 24 presumed non treponemal sera from cases of lepromatous lepra showed reactivity with cardchol antigen after heat inactivation at 56° C for 30 minutes 11 of these were still reactive after inactivation for 60 minutes and 8 were reactive even after 120 minutes at 56° C

None of the 24 presumed non-treponemal sera reacted with CLC antigen after inactivation at 56° C for 60 minutes, despite the fact that two of these sera were strongly reactive with CLC antigen after normal heat inactivation at 56° C for 30 minutes. All reactions of these sera with CLC except one were weaker than the corresponding reactions with cardehol.

TABLE 2

Examination of 32 Lepromatous Leprosy Sera in TPI and FTA200

	TPI		FTA200	
	No of sera	%	No of sera	%
Reactive	8	25	4	12 5
Non reactive	24	75	28	87 5
Total	32	100	32	100 0

Total presumed non treponemal sera (TPI and FTA200 non reactive) 24 (75%)
Total presumed treponemal sera (TPI and/or FTA200 reactive) 8 (25%)

Table 2 summarizes the results of the treponemal tests. The incidence of TPI reactivity (25 per cent) is almost the same as that found in the previous study by *Schmidt* of leprous sera from Madras (20 per cent) (14).

None of the patients had clinical lesions indicating treponemal disease. It was therefore considered justified to conclude that the 24 sera which did not react in the TPI or in the FTA200 were non-treponemal. Table 3 shows the reports of previous studies concerning the reactivity in complement fixation test with cardiolipin antigen in TPI non-reactive sera from cases of lepromatous leprosy.

TABLE 3

Comparison of Results in the Present Study with Serological Data in Previous Studies of TPI Non Reactive Cases of Lepromatous Leprosy Examined in Complement Fixation Test with Cardiolipin (CLC) and Cardehol (CC) Antigens

	Number of sera	Origin of sera	CLC reactive %	CC reactive %
Edmundson et al (1954)	182	Louisiana	64	
Kooij et al (1957)	?	South Africa	17	
Rosetti et al (1958)	51	Spain	14	
Fromm et al (1959)	289	Phillippines	4	
Schmidt (1959)	96	Egypt	13	62
Schmidt (1961)	88	Madras	20	53
Holst (1964)	24	Madras	13	67

The results obtained in the present study, *viz* the finding that less than 20 per cent of these sera were reactive in complement fixation test with ordinary cardiolipin antigen, are in good agreement with previous reports. The high incidence of reactivity reported by *Edmundson et al* (4) with this antigen has not been supported by later investigations.

TABLE 4

Effect of Heat Inactivation at 56° C for 30 60 and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin 24 TPI Non Reactive Sera from Cases of Lepromatous Leprosy

56°C inactivation	CF result	CLC antigen No of sera	CLC antigen %	CC antigen No of sera	CC antigen %
30 min	+	3	13	16	67
	—	21	87	8	33
60 min	+	0	0	11	46
	—	24	100	13	54
120 min	+	0	0	8	33
	—	24	100	16	67

The high incidence of reactivity with cardchol antigen previously demonstrated by *Schmidt* (13, 14) with TPI non-reactive lepromatous leprosy sera from Egypt and India was confirmed in the present study

The effect of prolonged heat inactivation can be seen from Tables 4 and 5 from the graphs in Figs 1 and 2

The average loss in reactivity during the first half hour of heat inactivation at 56° C has been shown previously (15) to be about 0 5 $\log_{10}$ value for presumed non-treponemal sera from cases of lepromatous leprosy

The present study shows that prolonged heat inactivation at 56° C

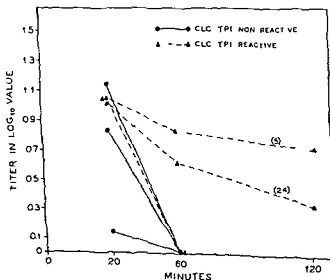


Fig 1

Variation in CLC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

None of the 24 presumed non-treponemal sera reacted with CLC antigen after inactivation at 56° C for 60 minutes, despite the fact that two of these sera were strongly reactive with CLC antigen after normal heat inactivation at 56° C for 30 minutes. All reactions of these sera with CLC except one were weaker than the corresponding reactions with cardchol.

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TABLE 4

Effect of Heat Inactivation at 56° C for 30, 60, and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin 24 TPI Non Reactive Sera from Cases of Lepromatous Leprosy

56°C inactivation	CF result	CLC antigen		CC antigen	
		No of sera	%	No of sera	%
30 min	+	3	13	16	67
	—	21	87	8	33
60 min	+	0	0	11	46
	—	24	100	13	54
120 min	+	0	0	8	33
	—	24	100	16	67

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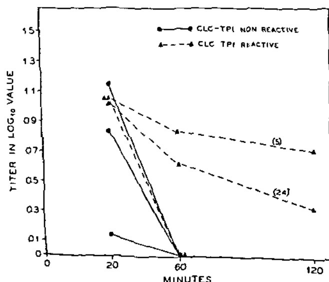


Fig 1

Variation in CLC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

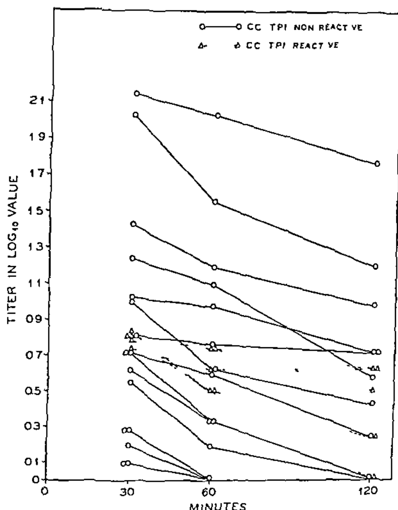


Fig 9
Variation in CC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

TABLE 5
Effect of Heat Inactivation at 56° C for 30, 60 and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin 8 TPI Reactive Sera from Cases of Lepromatous Leprosy

56°C inactivation	CF result	CLC antigen		CL antigen	
		No of sera	%	No of sera	%
30 min	+	3	38	5	62
	—	5	62	3	38
60 min	+	2	25	5	62
	—	6	75	3	38
120 min	+	2	25	4	50
	—	6	75	4	50

TABLE 6

Relation between Clinical Type of Lepromatous Leprosy and Reactivity with Cardiolipin Antigen in 24 Presumed Non Treponemal Cases

Type of lepromatous leprosy	Number of sera	Results with cardchol after heat inactivation at 56°C								
		30 min			60 min			120 min		
		Reactive		Mean log_{10} value	Reactive		Mean log_{10} value	Reactive		Mean log_{10} value
		No	%		No	%		No	%	
L 1	2	2	(100)	0.561	1	(50)	0.489	1	(50)	0.358
L 2	7	5	71	0.624	3	43	0.426	2	29	0.224
L 3	15	9	60	0.517	7	47	0.382	5	33	0.275
Total	24	16	67	0.552	11	46	0.403	8	33	0.275

TABLE 7

Relation between Duration of Leprosy Infection Symptoms and Reactivity with Cardchol Antigen in 24 Presumed Non Treponemal Sera fro Patients with Lepromatous Leprosy

Duration of symptoms (years)	Number of sera	Results with cardchol after heat inactivation at 56°C								
		30 min			60 min			120 min		
		Reactive		Mean log_{10} value	Reactive		Mean log_{10} value	Reactive		Mean log_{10} value
		No	%		No	%		No	%	
0.0- 1.0	8	6	75	0.456	4	50	0.301	2	25	0.179
1.1 5.0	8	7	88	0.686	4	50	0.444	3	38	0.325
5.1 22.0	8	3	38	0.513	3	38	0.465	3	38	0.322
Total	24	16	67	0.552	11	46	0.403	8	33	0.275

TABLE 8

Detailed Study of Effect of Duration of Heat Inactivation at 56° C on the Reactivity with CC and CLC Antigens of Two Lepromatous and Two Syphilitic Sera

	Lepromatous leprosy Osaka 3 log_{10} values		Lepromatous leprosy Coonor 4 log_{10} values		Secondary syphilis SERA 46 log_{10} values		Secondary syphilis SERA 51 log_{10} values	
	CLC	CC	CLC	CC	CLC	CC	CLC	CC
Unheated	1.575	2.052	ac	ac	1.384	1.288	1.193	1.288
56° C 2 min	1.193	2.338	0.859	1.527	1.384	1.145	1.264	1.145
56° C 5 min	1.097	1.926	0.239	1.527	1.336	1.145	1.431	1.145
56° C 10 min	0	2.004	0	1.455	1.288	1.169	1.312	1.169
56° C 20 min	0	1.885	0	1.312	1.431	1.193	1.217	1.217
56° C 30 min	0	1.718	0	1.193	1.288	1.145	1.241	1.193
56° C 45 min	0	1.394	0	1.097	1.288	1.002	1.193	1.050
56° C 60 min	0	1.241	0	0.787	1.288	1.002	1.193	1.169
56° C 120 min	0	0.954	ac	ac	1.288	0.954	1.145	1.050

ac — anticomplementary

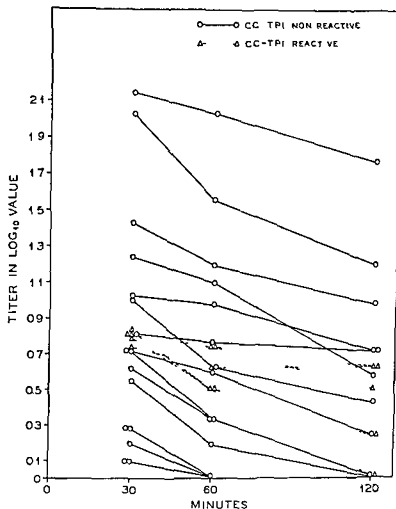


Fig 2

Variation in CC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

TABLE 5

Effect of Heat Inactivation at 56° C for 30, 60 and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin 8 TPI Reactive Sera from Cases of Lepromatous Leprosy

56°C inactivation	CI result	CLC antigen		CC antigen	
		No of sera	%	No of sera	%
30 min	+	3	38	5	62
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60 min	+	2	25	5	62
	—	6	75	3	38
120 min	+	2	25	4	50
	—	6	75	4	50

TABLE 6

Relation between Clinical Type of Lepromatous Leprosy and Reactivity with Cardchol Antigen in 24 Presumed Non Treponemal Cases

Type of lepromatous leprosy	Number of sera	Results with cardchol after heat inactivation at 56°C								
		30 min			60 min			120 min		
		Reactive		Mean log_{10} value	Reactive		Mean log_{10} value	Reactive		Mean log_{10} value
		No	%		No	%		No	%	
L 1	2	2	(100)	0 561	1	(50)	0 489	1	(50)	0 358
L 2	7	5	71	0 624	3	43	0 426	2	29	0 224
L 3	15	9	60	0 517	7	47	0 382	5	33	0 275
Total	24	16	67	0 552	11	46	0 403	8	33	0 275

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		30 min			60 min			120 min		
		Reactive		Mean log_{10} value	Reactive		Mean log_{10} value	Reactive		Mean log_{10} value
		No	%		No	%		No	%	
0 0- 1 0	8	6	75	0 456	4	50	0 301	2	25	0 179
1 1 5 0	8	7	88	0 686	4	50	0 444	3	38	0 325
5 1 22 0	8	3	38	0 513	3	38	0 465	3	38	0 322
Total	24	16	67	0 552	11	46	0 403	8	33	0 275

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	Lepromatous leprosy Osaka 3 log_{10} values		Lepromatous leprosy Coonor 4 log_{10} values		Secondary syphilis SERA 46 log_{10} values		Secondary syphilis SERA 51 log_{10} values	
	CLC	CC	CLC	CC	CLC	CC	CLC	CC
Unheated	1 575	2 052	ac	ac	1 384	1 288	1 193	1 288
56° C/ 2 min	1 193	2 338	0 859	1 527	1.384	1 145	1 264	1 145
56° C/ 5 min	1 097	1 926	0 239	1 527	1 336	1 145	1 431	1 145
56° C/ 10 min	0	2 004	0	1 455	1 288	1 169	1 312	1 169
56° C 20 min	0	1 885	0	1.312	1 431	1 193	1 217	1 217
56° C/ 30 min	0	1 718	0	1 193	1 288	1 145	1 241	1 193
56° C/ 45 min	0	1 384	0	1 097	1.288	1 002	1 193	1 050
56° C 60 min	0	1 241	0	0 787	1 288	1 002	1 193	1 169
56° C/120 min	0	0 954	ac	ac	1 288	0 954	1 145	1 050

ac — anticomplementary

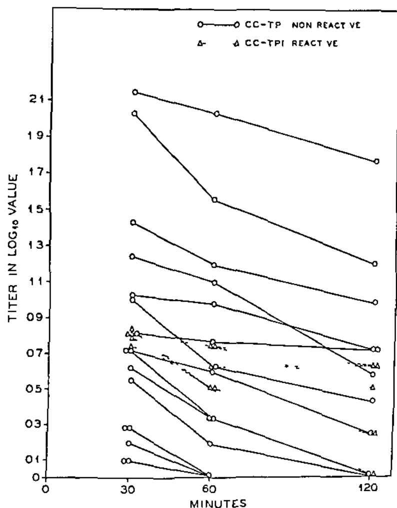


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56°C inactivation	CF result	ClC antigen		CC antigen	
		No of sera	%	No of sera	%
30 min	+	3	38	5	62
	—	5	62	3	38
60 min	+	2	25	5	62
	—	6	75	3	38
120 min	+	2	25	4	50
	—	6	75	4	50

and the type lepromatous lesion (L 1 L 2, L 3) (Table 6) nor does the heat stability of the reactions vary with the type of lepromatous leprosy

Table 7 indicates that the percentage of reactive sera is lower in patients with long standing infections However the reactivity in these sera seems to be influenced less by heat than the reactivity found in more recent cases ie of less than 5 years' duration The small number of sera in each group does not allow any final conclusions on this point

Table 8 gives the results of the supplementary study comparing the effect of heat inactivation of varying duration on the reactivity of two lepromatous and two syphilitic sera Fig 3 shows the variation in reactivity with the two antigens as a function of the duration of heat inactivation It is evident that the reacions of the two syphilitic sera from Copenhagen are more heat stable than those of the two lepromatous sera from Osaka and Coonor This is especially the case for the reactions with CLC antigen

CONCLUSION

Based on the assumption that the reactivity in complement fixation tests with CLC and cardchol antigens reflects the thermal stability of the immune globulins involved two conclusions can be drawn from these experiments

1) Cardchol antigen in lepromatous leprosy reacts with a relatively heat stable immune globulin with which the CLC antigen does not react (at the same time cardchol probably reacts with the heat labile immune globulin with which CLC antigen reacts)

2) Both the relatively heat stable and the heat labile antiipoidal immune globulin thus demonstrated in lepromatous leprosy differ in heat stability from the antilipoidal immune globulins demonstrated in the two sera from cases of secondary syphilis

SUMMARY

32 sera from cases of lepromatous leprosy were examined in complement fixation test with lecithin free cardiolipin antigen (cardchol CC)) as well as with standard cardiolipin antigen containing lecithin CLC) after heat inactivation at 56° C for 30 60 and 120 minutes respectively

On the basis of clinical examination and the results of the TPI 24 of these sera could be considered as originating from patients without concomitant treponemal infection

16 of the 24 TPI non reactive sera (67 per cent) were reactive with cardchol antigen but only three (13 per cent) reacted with CLC antigen after 30 minutes inactivation

Prolonged heat inactivation resulted in complete loss of reactivity with CLC antigen but 11 sera still showed reactivity with cardchol

results in a further decrease in reactivity during the second half hour This decrease is more pronounced for CLC antigen (0 7 log_{10} value) and less pronounced for cardchol antigen (0 2 log_{10} value)

Of the eight TPI-reactive sera, two showed an entirely different pattern (Nos 5 and 24), both reacting more strongly with CLC antigen than with cardchol antigen, and both reacting with CLC antigen after prolonged heat inactivation as well as with cardchol antigen Three TPI-reactive sera (Nos 9, 11 and 32), showed the same pattern of reactivity and heat stability in their reactions with the two antigens as that found with the presumed non-treponemal sera in the present study The remaining three sera in the TPI-reactive group (Nos 13, 14 and 31) were non-reactive with the two antigens in the complement fixation test

There is no apparent relation between the reactivity with cardchol

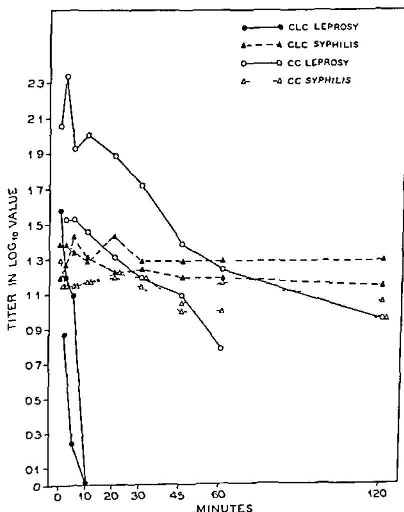


Fig 3

Variation in CLC and CC reactivity with duration of heat inactivation at 56° C for two lepromatous leprosy sera and two syphilitic sera

Acta path et microbiol scandinav 62, 377–380, 1964

The University of Bergen, School of Medicine, The Gade Institute,
Department of Microbiology,
and Broegelmann's Research Laboratory for Microbiology, Bergen, Norway

STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF *STAPHYLOCOCCUS AUREUS*

2 *Antigenic Formulas before and after Lysogenization*

By

TOR HOFSTAD

Received 9 iv 64

During a previous study of the agglutinogens in *Staphylococcus aureus* strains within the 80/81 complex (6), our type strains, and in particular the epidemic strain 263, went through repeated subcultures. The antigenic formulas and the phage patterns of strain 263 and other type strains both remained unchanged. The phenomenon of "antigenic loss variation" (14) was not encountered.

It has been shown in recent years (1, 3, 11, 12, 13) that the phage pattern of type 80 strains may be changed by lysogenization, *i.e.* by the introduction of a new phage into the strain by *in vitro* exposure of the strain to this phage, or presumably by contact with lysogenic, *i.e.* phage carrying, staphylococci in nature. Similar phage-induced conversion of some *Salmonella* strains has been followed by changes in somatic antigens (for references see (2)).

Therefore, before extending the studies of the antigenic structure of strain 263, it was found desirable to investigate in a controlled experiment whether or not the antigenic formula of this strain would change following lysogenization and repeated subculturing.

MATERIALS AND METHODS

Strain 263 has been described earlier (6). In addition, some selected strains (11), shown in Table 2, were included in part of the study. (These strains were kindly [illegible]

[illegible] ...en flooded on the surface [illegible] ...ded plates were allowed to [illegible] 1:10 and 1:100 from the [illegible]

Plates were incul[illegible]
Single colonies f[illegible]
and 81 and from [illegible]
in the lysis area [illegible]
typed. No second[illegible]

Standard meth[illegible] (16). The phages [illegible]

antigen after 60 minutes' inactivation, and 8 of these showed reactivity with cardehol antigen even after 120 minutes' inactivation at 56° C

A supplementary experiment showed that the antilipoidal immune globulins found in secondary syphilis differ in heat stability from those found in lepromatous leprosy if it is assumed that the heat stability of the reactions with the two antigens in complement fixation tests reflects the heat stability of the immune globulins themselves

Two of the eight TPI-reactive sera from cases of lepromatous leprosy show a heat stability of complement fixation reactions rather similar to that found for secondary syphilis Three other sera in this group behaved like the sera in the TPI non-reactive group, while the remaining three TPI-reactive sera were non-reactive with lipoidal antigens

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16 *Thom*[illegible] ung mit dem Serum Aussatziger Lepra [illegible] 910

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Therefore, before extending the studies of the antigenic structure of strain 263, it was found desirable to investigate in a controlled experiment whether or not the antigenic formula of this strain would change following lysogenization and repeated subculturing

MATERIALS AND METHODS

Strain 263 has been described earlier (6) In addition some selected strains (11), shown in Table 2 were included in part of the stud[illegible]
[illegible]

Immunization of rabbits agglutinin absorption and *agglutination* were carried out as described by Oeding (9) The techniques for preparation of the factor sera used were the same as in (6)

RESULTS

The phage patterns and antigenic formulas of six variant strains obtained by lysogenization of strain 263 are shown in Table 1 The strains are designated according to the phages used for lysogenization, for example 263-KS6-3 means variant number three obtained by lysogenization with the phage KS6

TABLE 1

Variant Strains Obtained by Lysogenization of Strain 263

Strain	Phage pattern	Antigenic formula
263	80/81/82/KS6	
263 80 1	80/81/82/KS6	
263 81-1	80	
263 81-2	52/52A/80/KS6	$a_3(k_2)$m 263 1 263 2
263 KS6 3	52/52A/80 82	
263 KS6-4	52/52A/81/82	
263-KS6 6	52/52A/80/82	

(k_2) – very weak

Other variant strains were isolated from the secondary growth in the lysis area of phage KS6, but they had all the typing pattern 52/52A/80/82 Strain 263-80-1 reacted weakly when typed with diluted phages

The variants were all typed with factor 262-1, 263-2, a_3, h_1, h_2, k_1k_2, k_1, and *m* sera The antigenic formulas were identical to that of the parent strain

The mother strain and the variants obtained were also examined for precipitinogens by double diffusion in agar While suspensions of the mother strain gave a weak, but distinct, polysaccharide A line (10), no such line was given by suspensions of the variant strains 263 KS6-3 and 363 KS6 6 This change, however, was only quantitative, as a weak polysaccharide A line could be obtained with concentrated extracts of the two variant strains (These examinations will be more fully dealt with in another paper)

The six variant strains were then subcultured daily on nutrient agar slopes Subcultures no 10, 20, 30, 40, and 50 were phage typed and examined in the above mentioned factor sera The antigenic formulas remained unchanged, and so also did the phage patterns with the exception of strain 263 81 2 When subculture no 50 had the same phage pattern as originally, subcultures no 10, 20, 30, and 40 were typed as 52/52A/80 82/KS6 (Routine Test Dilution (RTD) and RTD × 1 000)

Subculture no 50 of the six variant strains together with the parent strain were used for immunization of seven rabbits The seven strains exhausted each other's immune sera in cross-absorption experiments

Finally, *Rosenblum & Jackson*'s strains were typed with the same

factor sera as mentioned above (Table 2) With the exception of strains H101N (P60) and H101N(P53)(P60), all variants showed the same agglutinating patterns as the parent strains Factor h_2 serum could not be exhausted by nutrient agar or mannitol-salt agar cultures of the strains *H101N*, *H101N(B Wm)*, *H101N(P97)* and H101N(P53) grown at 37° C

TABLE 2

Phage Patterns and Antigenic Formulas of Selected Staphylococcus aureus *Strains*[1] *before and after Lysogenization*

Strains	Phage pattern	Antigenic formula
P184 P184(B Wm) P184(P53) P184(P97) P184(P53)(B Wm)	80/81/82/KS6 80/81/82/KS6 52,52A/80/81/82 52 52A/80/81/82 52/52A/80/81/82	a_3h_2 263 1 263 2
H101N H101N(B Wm) H101N(P97) H101N(P53)	52/52A/80/81/82 KS6 52/52A/80/81/82/KS6 52 52A/80/81/82 52/52A/80/81/82	a_3 263 1 263–2
H101N(P53)(P60) H101N(P60)	82 81/82/KS6	a_3h_2 263 1 263 2
P59 P59(P60)	52 52A/80/81/82/KS6 82/KS6	a_3h_2m 263 1 263 2
B Wm B Wm(P60)	52/52A/80/81/82 KS6 82/KS6	a_3h_2 263 1 263 2

[1] Parent strains and their phage variants from a study of *Rosenblum & Jackson* (11)

DISCUSSION

While the phage pattern of strain 263 was readily changed by lysogenization, the antigenic formula was not The alterations were, however, confined to gain or loss of sensitivity to phages within the 80 81 phage *complex* *This* is in keeping with the clear correlation that was shown in the previous study (6) between the major 263–1 antigen and phage group 1 staphylococci, but not between the 263–1 *antigen* and other agglutinogens and lysis by particular type phages

The stability of agglutinating pattern through subcultures is in accordance with studies by *Grun & Kuhn* (5) and *Kretzschmar & Kretzschmar* (8)

The gain of the heat-stable h_2 antigen *in* the variant strains H101N (P60) and H101N(P53)(P60) was unexpected The parent strain H101N was therefore lysogenized once again in the *same* manner as before (This was most kindly brought about by Professor *E D Rosenblum*) The h_2 antigen could not be demonstrated in the variant strains H101N(P60) and H101N(P53)(P60) now obtained Whether the presence of the h_2 antigen in the original variants H101N(P60) and H101N

Immunization of rabbits agglutinin absorption and *agglutination* were carried out as described by Oeding (9) The techniques for preparation of the factor sera used were the same as in (6)

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263 KS6 4	52/52A/81/82	
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The six variant strains were then subcultured daily on nutrient agar slopes Subcultures no 10, 20, 30, 40 and 50 were phage typed and examined in the above mentioned factor sera The antigenic formulas remained unchanged, and so also did the phage patterns with the exception of strain 263-81-2 When subculture no 50 had the same phage pattern as originally, subcultures no 10, 20, 30, and 40 were typed as 52/52A/80/82/KS6 (Routine Test Dilution (RTD) and RTD × 1 000)

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REDUCTION AND OXIDATION OF HYDROXYLAMINE BY EXTRACTS FROM *NEISSERIA MENINGITIDIS*

By

K JYSSUM and P E JONER

Received 8 ii 64

Meningococci contain a TPN dependent glutamic dehydrogenase An adaptation which is obviously necessary to obtain growth on a minimal medium with the ammonium ion as the only nitrogen source comprises an increased activity of a DPN dependent glutamic dehydrogenase (6, 7)

The literature dealing with the nitrate and nitrite reducing capacities of the Family *Neisseriaceae* has recently been reviewed by *Berger* (3) It seems to be generally agreed upon, that nitrate cannot be reduced by meningococci On the other hand, *Berger* (3) was able to demonstrate a reduction of nitrite in four meningococcal strains Two of these strains produced visible amounts of gas during the reaction No significant quantities of ammonia were produced

The present investigation was prompted by several observations concerning the effects of hydroxylamine in studies in which the energy metabolism of *Neisseria meningitidis* was the subject As the work advanced, it became apparent that the effects were due to enzyme reactions, some of which had not previously been described from heterotrophs

In the present communication two questions have been asked

1) May meningococcal extracts catalyse the reduction of hydroxylamine?
2) May meningococcal extracts catalyse the oxidation of hydroxylamine?

Hydroxylamine reduction has been reviewed by *Nason* (15) The Neurospora hydroxylamine reductase was demonstrated to be a pyridine nucleotide specific metalloflavoprotein which catalysed the stoichiometric reduction of hydroxylamine to ammonia (22) The corresponding pyridine nucleotide-specific metalloprotein from soy bean leaves showed a specific requirement for Mn^{++} ions (18) Manganese ions have also been found to be a highly specific activator for the flavin stimulated adaptive pyridine nucleotide hydroxylamine reductase of *Azoto-*

(P53) (P60) was an expression of the phenomenon of 'antigenic loss variation', or simply due to contamination, can only be guessed

The results obtained in this small scale experiment strengthen the view that the agglutinating pattern of a *Staphylococcus aureus* strain is a much more stable property than phage susceptibility

SUMMARY

The antigenic formula of the *Staphylococcus aureus* strain 263 (phage type 80 81/82 KS6) remained unchanged after lysogenization and repeated subculturing Similar results were obtained for some other staphylococcal strains within the 80 81 complex and their phage variants

The results indicate that the agglutinating pattern in *Staphylococcus aureus* strains within the 80 81 complex is a stable and reliable property

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commercially from Sigma Chemical Co Solutions of NH_2OH were prepared daily from $NH_2OH \cdot HCl$ purchased from E. Merck Darmstadt Germany The solutions were checked for the presence of nitrite before use since alkaline solutions are rapidly oxidized chemically

RESULTS

Reduction of Hydroxylamine

Hydroxylamine reductase activity was assayed by the following procedure The reaction mixture consisted of 1 44 $\times$ 10^{-4} M FMN 0 01 ml, 2 4 $\times$ 10^{-3} M TPNH 0 025 ml, 0 025 M G 6–P (sodium salt) 0 1 ml, 0 04 M $MnCl_2$ 0 01 ml, 3 $\times$ 10^{-4} M NH_2OH 0 2 ml, meningococcus extract dilution 0 05 ml and 0 1 M Tris buffer pH 7 4 to a total volume of 1 ml

The reaction was run at room temperature and stopped at the desired time by the addition of 0 5 ml of 1 3 per cent I_2 in glacial acetic acid The procedure of *Roussos & Nason* (18) was followed in order to determine the remaining hydroxylamine In these experiments a zero time value was obtained by the addition of I_2 plus glacial acetic acid to the mixture immediately after the reaction had been started Endogenous and non enzymatic decrease in hydroxylamine content was measured in parallel assays in which enzyme and electron donor system were omitted singly

From the results presented in Fig 1 it is seen that hydroxylamine rapidly disappears in the system described Since the meningococcal extracts used contain G 6-P dehydrogenase (8) the system described

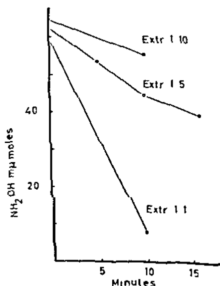


Fig 1

Disappearance of hydroxylamine in the presence of TPNH FAD and Mg^{++}
The effect of dilution of the meningococcus extract.

bacter (20) *Mager* (13) assumes that the TPNH specific hydroxylamine reductase and the TPNH specific sulphite reductase activities of *E coli* may well be catalysed by the same enzyme This author is of the opinion that the sulphite reduction represents the true physiological function of the enzyme

The hydroxylamine reductases in many denitrifying bacteria do not seem to operate with DPNH or TPNH as electron donors This is the case with *Micrococcus denitrificans* (11) as well as with *Pseudomonas denitrificans* (21) The major difference between the Pseudomonas reductase and that of Micrococcus appears to be the absence of FAD requirement from the latter

Oxidation of hydroxylamine has so far only been reported from the chemolithotrophic bacterium Nitrosomonas This area of nitrification has been reviewed by *Aleem & Nason* (2) Cell free preparations from *Nitrosomonas europea* have been shown to oxidize hydroxylamine to nitrite when mammalian cytochrome C is used as the electron acceptor (5, 16) An unstable intermediate is apparently formed during the oxidation of hydroxylamine to nitrite This may be NOH (4, 5) The experiments of *Aleem et al* (1) were taken to suggest that nitrohydroxylamine is an intermediate in the oxidation of hydroxylamine, formed by the initial oxidative condensation of hydroxylamine and nitrite

MATERIALS AND METHODS

Meningococcal strains The present study was performed with the virulent *Neisseria meningitidis* strain M6 The serologic nature of this strain has been reported previously (6) The strain was arbitrarily chosen as the main test microbe The bacteria were maintained in the lyophilized state When not otherwise stated the strain was adapted to growth on a minimal medium (6)

Cell free extracts The methods used for growth harvesting and extraction after ultrasonic disintegration of the cells have been described previously (10)

Analytical procedures Hydroxylamine was determined according to the procedure described by *Roussos & Nason* (18) which involves the oxidation of hydroxylamine by iodine to nitrite This procedure permits the determination of hydroxylamine in mμmole quantities Nitrite was measured by the method of *Medina & Nicholas* (14) Ammonia was determined by the Conway microdiffusion technique in conjunction with the phenolhypochlorite test for ammonia according to the procedure of *Russel* (19) Protein was analysed by the method of *Johnson* in the way it has previously been used (9)

Equipment Spectrophotometry was performed either with a Hilger and Watts Uvispec spectrophotometer or in a Beckman DB spectrophotometer In both instruments analyses were performed with 1 cm light paths

Cofactors and chemicals Mammalian cytochrome C (Sigma type III from horse heart) was oxidized by means of acidification with 1 N HCl to pH 3 and neutralization with an equivalent amount of alkali when the band at 550 mμ had vanished usually after 30 to 45 minutes at room temperature Reduced cytochrome C was obtained by reduction with Pd H_2 A neutral cytochrome C solution mixed with approximately 0 2 g palladium asbestos (5 per cent) per 10 ml was first gassed with N_2 for 5 minutes and then with H_2 for 1 hour and again with N_2 for 5 minutes The extent of reduction was tested by measuring the ratio of the absorbancy of the solution at 550 mμ to that at 565 mμ The reduction of cytochrome C was essentially calculated according to the method of *Nielsen & Lehninger* (17) in terms of ½ Δ FeIII cytochrome C mμmoles (equal to mμmoles 0) The pyridine nucleotide cofactors oxidized as well as reduced the flavin cofactors and the G 6 P were also obtained

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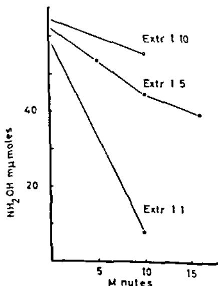


Fig 1

Disappearance of hydroxylamine in the presence of TPNH FAD and Mg^{++} The effect of dilution of the meningococcus extract

was able to maintain almost all the added pyridine nucleotide in the reduced state in the presence of G-6-P as electron donor

The cofactor requirement of the system presented was analysed in the way it has been recorded in Table 1

TABLE 1

The Effect of Various Cofactors and Electron Donors on the Hydroxylamine Reduction by Extracts from Neisseria meningitidis

Experiment no	Factor omitted from the system	NH_2OH at the start mμmoles	NH_2OH recovered after 10 min mμmoles
I	None	47.5	20.0
	FMN	47.5	34.0
	TPNH	47.5	25.6
	G 6-P	47.5	23.1
	Mn^{++}	47.5	42.7
II	None	50.4	21.8
	TPNH and G 6 P	50.4	43.1
	G 6 P and Mn^{++}	50.4	48.8
	TPNH and Mn^{++}	50.4	49.0
	FMN and G 6 P	50.4	35.5
	FMN and TPNH	50.4	29.6
	FMN and Mn^{++}	50.4	47.9
III	None	55.6	21.3
	TPNH G 6 P and Mn^{++}	55.6	55.0
	FMN G 6 P and Mn^{++}	55.6	52.0
	FMN, TPNH and Mn^{++}	55.6	52.3
	FMN, TPNH and G 6-P	55.6	47.3
	FMN TPNH G 6 P and Mn^{++}	55.6	55.6
	Extract	55.6	52.5

The experimental data show that Mn^{++} is essential for the reaction It is also seen that either TPNH or G 6 P must be added as a source of electrons Also, there seems to be a significant increase in hydroxyl amine disappearance when FMN is added to the system

In following series of experiments it was found that DPNH could replace TPNH in the system described There is some indication, however, that the latter is slightly more effective as electron donor in the meningococcal system

The DPNH and TPNH oxidases of *Neisseria meningitidis* are very active (8, 9) and compete strongly for reduced pyridine nucleotide The stimulatory effect of TPNH on the rates of hydroxylamine reduction is shown in Table 1 With a view to the interfering oxidase activities it may be understood why it was not possible to do stoichiometric or kinetic studies based on the rate of oxidation of the reduced pyridine nucleotides when these were used as the only electron donors

The hydroxylamine reductase in crude extracts from *Neisseria men-*

ingitidis could be stored for at least 14 days at 18° C without appreciable loss of activity

The disappearance of hydroxylamine from the assay system was accompanied by ammonia formation This was demonstrated in a reaction system similar to that previously described with the exception that a phosphate buffer was used instead of the Tris buffer The procedure was essentially as that described by *Zucker & Nason* (22) Endogenous and non enzymatically formed ammonia was measured in parallel assays A corresponding zero time control was also included In such an experiment 2 µg NH_3 was recovered where a quantitative recovery of the hydroxylamine disappeared should have given 3 4 µg NH_3

Oxidation of Hydroxylamine

Cell free preparations from *Neisseria meningitidis* actively catalyse the reduction of mammalian cytochrome C when hydroxylamine is used as the electron donor This activity was first measured in a system with the following components Oxidized cytochrome C 0 06–0 07 µmoles, NH_2OH 10 µmoles 0 1 M Tris buffer pH 7 2 to a total volume of 2 ml The experiments were performed at room temperature and followed by the reading of absorbancy at 550 mµ In Fig 2 the results from some experiments in this technique have been presented

Neisseria meningitidis contains an active cytochrome C oxidase which also oxidizes reduced mammalian cytochrome C in the way it has been

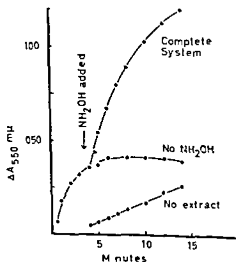


Fig 2

Reduction of mammalian cytochrome C by hydroxylamine in the presence of meningococcus extract
Cytochrome C added at zero time hydroxylamine as shown

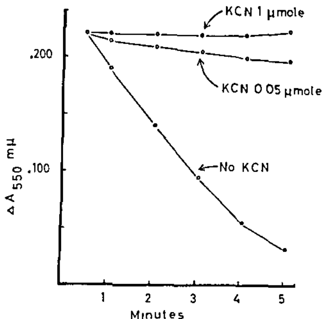


Fig 3

Oxidation of reduced mammalian cytochrome C by meningococcus extract Inhibition by cyanide

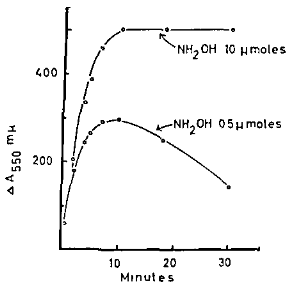


Fig 4

Reduction of mammalian cytochrome C by hydroxylamine in the presence of meningococcus extract Reoxidation by cytochrome oxidase

shown by the experiments recorded in Fig 3 This cytochrome oxidase effect becomes apparent in the assay of hydroxylamine oxidation When small quantities of substrate were used, prolonged incubation resulted in a reoxidation of the cytochrome This effect has been demonstrated in Fig 4 It is of course desirable to be able to study the rate of reduction of cytochrome without the competition from the oxidase Accord-

ingly, the effect of the conventional oxidase inhibitor cyanide on the experimental system was investigated. Unfortunately also the rate of reduction of cytochrome C which is mediated by hydroxylamine is to some extent inhibited by this chemical.

TABLE 2

The Effect of Cyanide on the Reduction of Mammalian Cytochrome C by Hydroxylamine in the Presence of Extracts from Neisseria meningitidis

	Reduction of cytochrome C ½ Fe^{III} cytochrome C mμmoles	
	Cyanide 1 μmole	No cyanide
NH_2OH as the substrate	9.7	13.0
Endogenous metabolism	6.5	5.9
Reduction due to NH_2OH	3.2	7.1

Reduction of cytochrome C was calculated after 5 minutes.
Chemical reduction from NH_2OH deducted.

From some data presented in Table 2 it is seen that cyanide in the concentration used inhibits the reaction under study approximately 50 per cent. Obviously, the endogenous reduction of cytochrome C is not similarly inhibited. When the oxidase is blocked, we find an increased endogenous reduction, presumably because the reoxidation is eliminated. With 1 μmole cyanide the hydroxylamine mediated cytochrome C reduction was 50 to 55 per cent inhibited. With 0.1 μmole cyanide, however, the reduction such as measured was no more than 5 per cent inhibited while the cytochrome oxidase was virtually stopped. The quantity 0.1 μmole has accordingly been chosen for the following experiments in which cyanide was deemed necessary.

Fluoride in a quantity of 10 μmoles did not interfere with the rate of reduction of cytochrome with hydroxylamine.

The reduction of cytochrome C by hydroxylamine is not dependent on P_i. On the contrary, the addition of 3 μmoles P_i resulted in approximately 20 per cent inhibition of the reduction rate when [illegible]

[illegible]

[illegible] extract was incubated with hydroxylamine and cytochrome C under atmosphere of nitrogen a gas was formed. The composition of this gas has not been analysed.

The hydroxlamine mediated reduction of cytochrome C in meningococcal extracts is still present after dialysis for 22 hours against Tris buffer. Extracts may be kept at −18° C for more than 14 days without significant loss of activity.

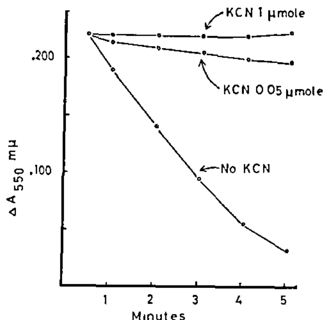


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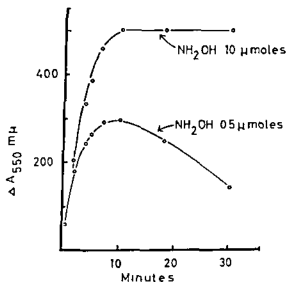


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has not previously been found in heterotrophs The reaction has been discussed in relation to that found in *Nitrosomonas europea*

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The first activity is assumed to be the result of a hydroxylamine reductase in the meningococcal extracts, corresponding to those described from other microbes. The enzyme is similar to that found in soy bean (22) since manganese ions serve as an activator and either FAD or *FMN meets the flavin requirement.* Like the enzymes from soy bean and Neurospora the reaction product appears to be ammonia. In *Neisseria meningitidis* the hydroxylamine reductase seems to be a constitutional enzyme.

The second activity which is considered in this paper may be assumed to signify a hydroxylamine oxidase. In any case, mammalian cytochrome C is reduced by hydroxylamine in the presence of cell free extracts from *Neisseria meningitidis*. The reaction product of this system has not been identified, but it is neither nitrite, nor nitrate. The product may turn out to be an unstable intermediate like NOH which is non-enzymatically oxidized to for instance N_2O (12).

The reaction found in *Neisseria meningitidis* seems in many respects to be similar to that found in *Nitrosomonas europea* (5). In contrast to that system, however, it has not been possible to demonstrate a synthesis of nitrite in the meningococcal system, even in the absence of cyanide. The cytochrome C reduction is approximately 50 per cent inhibited by 5×10^{-4} M cyanide. This inhibition may be due to the formation of a chelate with a metal cofactor. Such a cofactor has not been identified. The quantity of cyanide necessary to inhibit the cytochrome oxidase of *Neisseria meningitidis* 100 per cent does not inhibit the cytochrome reduction from hydroxylamine more than approximately 5 per cent.

The hydroxylamine-cytochrome C reductase of *Neisseria meningitidis* seems to be a constitutional enzyme.

SUMMARY

Meningococcal cell free extracts possess an activity corresponding to a hydroxylamine reductase. The reaction utilizes reduced pyridine nucleotides as electron donors and requires added flavin nucleotides for maximal activity. Mn^{++} is a specific activator. The product of the reaction appears to be ammonia.

Meningococcal cell free preparations also have been shown to reduce mammalian cytochrome C in the presence of hydroxylamine. The reaction is assumed to signify a hydroxylamine oxidase, an enzyme wich

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RESULTS

The reduction of mammalian cytochrome C which is catalysed by extracts from *Neisseria meningitidis* does not seem to need specific cofactors. An inhibition by fairly high concentrations of cyanide however may indicate a metal cofactor requirement (6).

A study of phosphorylation calls for the addition of ADP and P_i to the mixture. Also the addition of Mg^{++} seems to be required by all known phosphorylations coupled to an electron flux to cytochrome C. Accordingly the influence of these factors on the hydroxylamine mediated reduction of cytochrome C in meningococcal extracts was investigated.

From some data presented in Table 1 it is seen that the addition of each of the three factors alone inhibits the degree of reduction when measured over a period of five minutes.

TABLE 1

The Inhibition of Hydroxylamine Mediated Reduction of Cytochrome C by Mg, P_i and ADP

Substance added	Quantity	Per cent activity
None		100 0
P_i	3 2 µmoles	78 7
ADP	5 0 µmoles	14 5
Mg	10 0 µmoles	33 2
Mg	5 0 µmoles	40 6

Activity was calculated in terms of Δ 550 mµ wave per minute over an observation time of five minutes. Endogenous and chemical reduction deducted.

According to these data it appears natural to reduce the concentration of ADP and Mg as much as possible. On the other hand the quantities of these reagents should be chosen so as to limit the endogenous incorporation of P. Since a major part of this incorporation is due to the polynucleotide phosphorylase (5) the quantities selected should be unfavourable for this enzyme reaction. For the present studies 5 µmoles Mg were used giving a concentration of 2 5 mM. This is still far from the optimal concentration for the polynucleotide phosphorylase which is approximately 1 mM (5). The quantity of ADP was reduced to 2 5 µmoles an amount which when added alone still inhibits the reduction of cytochrome C. A total of 1 6 µmoles P_i was added to the system. This results in a ratio ADP to P_i of 1 56 which is still far from the ratio 0 78 found optimal for the polynucleotide phosphorylase (5).

When the mixture of Mg, ADP and P_i finally decided upon was added to the system the degree of reduction of cytochrome C was increased rather than the opposite. In Fig 1 the time course curves from such an experiment are presented. When considering the linearity of the supplemented system during the first three to four minutes of observation as opposed to the curve representing the basal system it be

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PHOSPHORYLATION COUPLED TO THE REDUCTION OF CYTOCHROME C BY HYDROXYLAMINE IN EXTRACTS FROM *NEISSERIA MENINGITIDIS*

By

K Jyssum and P E Joner

Received 17 iv 64

Meningococcal cell free extracts have been found to possess an activity corresponding to a hydroxylamine reductase This reaction utilizes reduced pyridine nucleotides as electron donors and requires manganese ions FMN must be added for maximal activity The product of the reaction appears to be ammonia (6)

Meningococcal cell free preparations also have been found to reduce mammalian cytochrome C in the presence of hydroxylamine The reaction is assumed to signify a hydroxylamine oxidase The reaction product is neither nitrite, nor nitrate (6)

In the chemolithotrophic *Nitrosomonas europea*, the only microbe from which a similar type of reaction, so far, has been reported, the oxidation of hydroxylamine is thought to be an important donor of energy (7) There is also some indication that phosphorylation may take place in the Nitrosomonas system (2)

In the present paper one question has been asked Is the reduction of mammalian cytochrome C by hydroxylamine in the presence of meningococcal extracts coupled with a phosphorylation of ADP to ATP?

The use of added ferricytochrome C as electron acceptor in studies of oxidative phosphorylation was briefly reported by *Slater* (11) The methods, however, were elaborated by *Nielsen & Lehninger* (8) and of *Borgstrom, Sudduth & Lehninger* (1)

MATERIALS AND METHODS

The methodology and experimental manipulations employed in this investigation were analogous to those previously used (6) The test microbe was the meningococ

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Substance added	Quantity	Per cent activity
None		100.0
P	3.2 μmoles	78.7
ADP	5.0 μmoles	14.5
Mg^{++}	10.0 μmoles	33.2
Mg	5.0 μmoles	40.6

Activity was calculated in terms of $\Delta A_{550\,m\mu}$ average per minute over an observation time of five minutes. Endogenous and chemical reduction deducted.

According to these data it appears natural to reduce the concentration of ADP and Mg^{++} as much as possible. On the other hand the quantities of these reagents should be chosen so as to limit the endogenous incorporation of P_i. Since a major part of this incorporation is due to the polynucleotide phosphorylase (5) the quantities selected should be unfavourable for this enzyme reaction. For the present studies 5 μmoles Mg^{++} were used giving a concentration of 2.5 mM. This is still far from the optimal concentration for the polynucleotide phosphorylase which is approximately 1 mM (5). The quantity of ADP was reduced to 2.5 μmoles, an amount which when added alone still inhibits the reduction of cytochrome C. A total of 1.6 μmoles P was added to the system. This results in a ratio ADP to P_i of 1.56 which is still far from the ratio 0.78 found optimal for the polynucleotide phosphorylase (5).

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The methodology and experimental manipulations employed in this investigation were analogous to those previously used (6) The test microbe was the meningococcus strain M6 maintained as previously described

Determination of orthophosphate uptake The phosphate uptake was determined

incorporation is due to the activity of the polynucleotide phosphorylase and the adenylate kinase (5) A heavy incorporation, however, may also be caused by other exchange reactions independent of electron transport This type of exchange is strongly influenced by several conventional inhibitors (*Jyssum & Jyssum* unpubl results) It is therefore clear that the endogenous uptake of inorganic phosphate must be studied in great detail in order to permit any definite conclusions to be drawn regarding a phosphorylation coupled to the electron flux.

In the present experiments the endogenous phosphorylation had to be considered under two different conditions The first is concerned with cytochrome C when added alone The other with hydroxylamine when present but without an added electron acceptor

Perusal of the data presented in Table 2 shows that the addition of cytochrome C results in a pronounced endogenous reduction This electron flow is accompanied by a small and variable uptake of inorganic phospnate We will call this an "endogenous cytochrome phosphorylation"

TABLE 2

Reduction of Ferricytochrome C and Incorporation of P_i in the Presence of Meningococcal Extracts
Analysis of the Endogenous Metabolism and of Hydroxylamine Effects

	Exper no		1	2	3
Endogenous Metabolism mμmoles	P_i Uptake	Extr + Cyto	25 2	25 4	40 5
		Extr	23 8	25 4	38 5
		Effect of Cyto	1 4	–	2 0
	Red of Cytochrome		11 5	11 8	7 8
Hydroxylamine Metabolism mμmoles	P_i Uptake No Electron Flux to Cytochrome	Extr + NH_2OH	24 3	26 4	42 1
		Extr	23 8	25 4	38 5
		Effect of NH_2OH	0 5	1 0	3 6
	P_i Uptake Electron Flux to Cytochrome	Extr + Cyto + NH_2OH	30 8	28 8	46 2
		Extr + Cyto	25 2	25 4	40 5
		Effect of NH_2OH	5 6	3 4	5 7
	P_i Uptake due to NH_2OH Mediated Reduction of Cytochrome		5 1	2 4	2 1
	Red of Cytochrome due to NH_2OH *		4 2	5 5	2 9
	P O Ratio		1 2	0 4	0 7

* Hydroxylamine mediated reduction was calculated by subtracting the sum of endogenous reduction and chemical reduction

Th

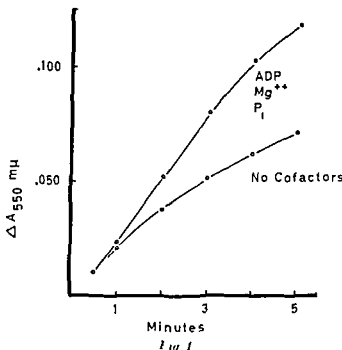


Fig 1

The influence of a combined addition of ADP, Mg^{++} and P_i on the reduction of cytochrome C by hydroxylamine in the presence of meningococcus extract

comes apparent that the effect of the factors added is not on the initial rate of the reaction, but on the faculty of the system to maintain a high initial rate of reduction over a considerable period of time In other words, a rate limiting factor appearing very early in the basal system has been eliminated or rather significantly postponed as the result of a combined addition of Mg^{++}, ADP and P_i

After the establishment of this experimental system studies concerning the incorporation of inorganic orthophosphate were started The type of experiments recorded in Table 2 was performed in order to obtain a "*balance sheet*" *concerning the incorporation of* P_i *during endogenous* electron flow, in the presence of hydroxylamine without electron flow to cytochrome C, and during electron flow caused by hydroxylamine Since we primarily were interested in the electron transport to cytochrome C cyanide was added in order to inhibit the cytochrome oxidase such as previously described (6)

The use of cyanide in such experiments is open to some discussion Cyanide forms a complex with cytochrome C (3) It is assumed, however, that the rate of this reaction with the cyanide concentration chosen, *is too slow to inhibit the rate of reduction of cytochrome C in* the short intervals studied

Previous investigations concerning the incorporation of $32P_i$ in the presence of extracts from *Neisseria meningitidis* have demonstrated considerable incorporation independent of electron flux The radioactivity is located mainly in ADP and ATP under the present experimental and analytical conditions A major part of this "endogenous"

incorporation is due to the activity of the polynucleotide phosphorylase and the adenylate kinase (5). A heavy incorporation however may also be caused by other exchange reactions independent of electron transport. This type of exchange is strongly influenced by several conventional inhibitors (*Jyssum & Jyssum* unpubl. results). It is therefore clear that the endogenous uptake of inorganic phosphate must be studied in great detail in order to permit any definite conclusions to be drawn regarding a phosphorylation coupled to the electron flux.

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	Exper. no.		1	2	3
Endogenous Metabolism mμmoles	P_i Uptake	Extr. + Cyto.	25.2	25.4	40.5
		Extr.	23.8	25.4	38.5
		Effect of Cyt.	1.4		2.0
	Red. of Cytochrome		11.5	11.8	7.8
Hydroxylamine Metabolism mμmoles	P_i Uptake No Electron Flux to Cytochrome	Extr. + NH_2OH	24.3	26.4	42.1
		Extr.	23.8	25.4	38.5
		Effect of NH_2OH	0.5	1.0	3.6
	P_i Uptake Electron Flux to Cytochrome	Extr. + Cyto. + NH_2OH	30.8	28.8	46.2
		Extr. + Cyto.	25.2	25.4	40.5
		Effect of NH_2OH	5.6	3.4	5.7
	P_i Uptake due to NH_2OH Mediated Reduction of Cytochrome		5.1	2.4	2.1
	Red. of Cytochrome due to NH_2OH		4.2	5.5	2.9
	P/O Ratio		1.2	0.4	0.7

Hydroxylamine mediated reduction was calculated by subtracting the sum of endogenous reduction and chemical reduction.

The complete system contained ADP
KCN 0.1 μmoles Ferricytochrome C
Meningococcus extract dilution 0.2 ml
of 2 ml. The
cer

Since hydroxylamine, the substrate of the present study, is an inhibitor of several enzyme reactions in the concentration actually used an effect on the endogenous P_i incorporation has to be considered From the data presented in Table 2 it is seen that the endogenous incorporation of P_i is in fact increased in the presence of hydroxylamine even when an electron acceptor is absent This phosphorylation will will be referred to as the "endogenous hydroxylamine phosphorylation

The experiments presented in Table 2 may on the whole be taken as an indication that a phosphorylation indeed occurs concomitant with the hydroxylamine mediated reduction of cytochrome C

TABLE 3

Effects of Various Treatments and Chemical Agents on Phosphorylation Associated with Hydroxylamine Mediated Reduction of Cytochrome C in Meningococci

Exper no		1	2	3	4	5	6	7	8
Treatment of Extract		–	–		Ageing	–		Dialysis	Ageing
Chemical Agent Added *		–	DNP	As	–	F	F	–	F
Endogenous Metabolism mμmoles	P_i Uptake due to Cytochrome	3 7	4 1	0 8	4 9	–	0 9	–	–
	Reduction of Cytochrome	15 7	11 9	9 2	5 8	11 7	3 1	2 1	5 2
Hydroxylamine Metabolism mμmoles	P_i Uptake due to NH_2OH No Electron Flux to Cytochrome	3 8	4 5	0 9	7 5	2 0	7 8	6 9	2 8
	P_i Uptake due to NH_2OH Electron Flux to Cytochrome	8 9	9 1	0 7	2 6	1 8	8 6	6 7	3 1
	P_i Uptake due to NH_2OH Mediated Red of Cytochrome	5 1	4 6				0 8	–	0 2
	Reduction of Cytochrome due to NH_2OH	2 9	2 4	3 9	7 2	1 0	4 1	2 4	5 0
	P O Ratio	1 7	1 9				0 2		

* ... ·9 + M
3 M
× 10 + M

The experimental system and calculations were analogous to those described in the legend of Table 2

Common to systems in which phosphorylations are coupled to electron transport "above" substrate level is the capacity to become uncoupled. Perhaps the least drastic among the classical uncoupling procedures is ageing. In Table 3 the effect of ageing has been investigated among some other uncoupling procedures.

Evidently the phosphorylation coupled to the reduction of cytochrome C from hydroxylamine is completely uncoupled. On the other hand, the reduction is increased rather than reduced. The latter observation is in agreement with previous findings concerning the stability of the electron transport system in question (6).

A comparison of the coupled system with the uncoupled one permits conclusions to be drawn regarding the nature of the two kinds of endogenous phosphorylation previously referred to.

The endogenous cytochrome phosphorylation may on the one hand be considered a true electron transport phosphorylation. On the other hand, it may be the result of an increased endogenous phosphorylation on the substrate level due to a reestablishment of the electron flow. From the data presented in Table 3 it is seen that the endogenous cytochrome phosphorylation is not uncoupled after ageing of the extract. The endogenous effect of cytochrome C is accordingly not interpreted as an electron transport phosphorylation.

The endogenous hydroxylamine phosphorylation may be an effect independent of electron flux. Another explanation would be that it is due to an electron flux which is independent of added cytochrome C. Such an electron flow might be due to an endogenous pool of oxidized cytochrome C, or electrons might somehow escape the cyanide block established. Studies with aged extracts clearly demonstrate that the endogenous hydroxylamine phosphorylation is unimpaired by this treatment in the way it has been illustrated in Table 3. The endogenous hydroxylamine phosphorylation is accordingly construed as an effect unrelated to electron flux phosphorylation.

In successive series of experiments some conventional inhibitors and uncoupling agents were tested. In the experiments recorded in Table 3 one observation has been made which is in disagreement with the general experience from electron transport phosphorylation in bacteria.

The phosphorylation is obviously strongly to completely inhibited by the addition of 10 μmoles fluoride without any significant effect on the reduction of cytochrome C. Fluoride in this concentration inhibits the endogenous cytochrome phosphorylation without a similar effect on the endogenous hydroxylamine phosphorylation. Obviously, the mechanisms involved in the two endogenous effects are entirely different. Arsenate which also uncouples the phosphorylation due to a reduction of cytochrome C by hydroxylamine, seems to reduce the endogenous cytochrome phosphorylation as well as the endogenous hydroxylamine phosphorylation.

DISCUSSION

A reduction of ferricytochrome C is mediated by hydroxylamine in the presence of extracts from *Neisseria meningitidis*. When the experimental system is supplemented with Mg^{++}, ADP and labelled orthophosphate the reduction is accompanied by a phosphorylation.

In the experiments performed in order to demonstrate this effect the reoxidation of ferrocytochrome C was prevented by an inhibition of the cytochrome oxidase with cyanide. With the cyanide concentration chosen the reduction of cytochrome by hydroxylamine was approximately 5 per cent inhibited (6).

The experimental system which was used in the present investigation was arranged in order to obtain maximal reduction of cytochrome C with a minimum of endogenous uptake of radioactive orthophosphate. No special effort was made to establish optimal conditions for a coupled phosphorylation.

The data presented are consistent with the view that phosphorylation is coupled to the transport of electrons which is mediated by hydroxylamine. It is conceivable, however, that the effects observed may be secondary to some interference of hydroxylamine with the endogenous metabolism of the extract used. The chemical might be thought to change the endogenous flow of electrons, thus canalizing them through a phosphorylating chain to cytochrome C. Several attempts have been made to demonstrate such an effect on the flow of electrons from DPNH, TPNH or succinate to cytochrome C, but without success. In this connection it is relevant to mention experiments which clearly demonstrate that reduction of cytochrome C by DPNH, TPNH and succinate under the conditions of the present experiments occurs without a concomitant phosphorylation (*Jyssum & Jyssum* unpubl. results). In the experiments reported in this paper the endogenous cytochrome phosphorylation was found to be unaffected by ageing of the extract (Table 3). It has been stated above that this is taken to indicate that the endogenous electrons, like those from the electron donors just mentioned, do not contribute to an electron transport phosphorylation.

The phosphorylation which is associated with the reduction of cytochrome C by hydroxylamine was uncoupled by ageing of the extract, by the addition of arsenate, and by the addition of fluoride ions, but not by DNP in the concentrations usually employed.

The effects of uncoupling agents on bacterial systems have recently been reviewed by *Ishikawa & Lehninger* (4). The lack of uncoupling by DNP has been reported from certain other, but not all, microbial systems. Fluoride ions do not uncouple oxidative phosphorylation in mitochondria. *Siekevitz & Potter* (10), however, found that if mitochondria are preincubated in the presence of F^-, they become inactive with respect to phosphorylation. These authors assume that F^- binds some compound or compounds necessary for mitochondrial phospho-

rylation The lack of effect in short time experiments with mitochondria may indicate that the intact structures are to some extent protected against the fluoride effect Findings by *Pullman, Penefsky, Datta & Racker* (9) that the intramitochondrial Mn^{++} activated ATP-ase to some extent was inhibited in the presence of 0 02 M fluoride may also indicate an effect of this inhibitor on oxidative phosphorylation

Whether the findings from the present experiments with meningococcal extracts indicate a true difference between the hydroxylamine-cytochrome C reductase system and other bacterial systems concerned with electron transport phosphorylation is not known

The analysis of uncoupling agents has not been considered a main issue of the present investigation The experiments reported were performed with the purpose of exploring the nature of the effects previously called "endogenous cytochrome phosphorylation" and "endogenous hydroxylamine phosphorylation" (Table 2) The results are taken to indicate that these effects are unrelated to an electron transport phosphorylation

The P O ratios calculated in the present paper are open to some discussion A decisive point in this discussion is the endogenous reduction of cytochrome C A calculation of hydroxylamine mediated reduction by subtracting the sum of endogenous and chemical reduction may result in too low values It may be argued that the endogenous reduction is decreased in the presence of hydroxylamine, thus only unfavourably competing for the common pool of added cytochrome C If so, the P O ratios calculated may be too high

The P O ratios calculated in the present experiments are consistent with the conclusion that a maximum of two phosphorylations are coupled to the transport of each pair of electrons to cytochrome C from hydroxylamine

SUMMARY

The reduction of mammalian ferricytochrome C which is mediated by hydroxylamine in the presence of extracts from *Neisseria meningitidis* is accompanied by a phosphorylation

In these experiments the reoxidation of ferrocytochrome C was prevented by an inhibition of the cytochrome oxidase with cyanide The system was supplemented with labelled orthophosphate, ADP and Mg^{++}

The phosphorylation connected with the reduction of cytochrome C from hydroxylamine has been analysed in relation to the endogenous uptake of inorganic phosphate

P O ratios have been calculated from measurements of the amounts of ferricytochrome C reduced and the orthophosphate taken up The ratios calculated have been discussed in relation to the endogenous electron flow to cytochrome C

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INACTIVATION OF METHICILLIN, OXACILLIN, CLOXACILLIN, AND CEPHALOTHIN BY STAPHYLOCOCCAL PENICILLINASE

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The so called penicillinase-resistant penicillins are not quite unaffected by penicillinase It was shown already in early reports on methicillin that this compound is inactivated by penicillinase produced by *B cereus* (16) and several authors have found that methicillin, when present in high concentrations is inactivated at an appreciable rate also by staphylococcal penicillinase (15, 18, 20) In concentrations which can be achieved in the human organism during its therapeutic use, methicillin is generally believed to be unaffected by staphylococcal penicillinase

Consequently, the slightly lower susceptibility to methicillin of penicillinase producing as compared to penicillin sensitive staphylococci, observed by several authors is not regarded as a manifestation of penicillinase activity (4)

The newer penicillins e g the isoxazolyl compounds, and some of the cephalosporins are less resistant than methicillin to the action of staphylococcal penicillinase and they are slowly inactivated also when present in 'therapeutic concentrations The fact that these compounds are much less active against a large than against a small inoculum of a penicillinase producing *Staphylococcus aureus*, and that non penicillinase producing staphyloccoci generally are more sensitive than penicillinase producing strains has been explained as a result of inactivation (4 13)

Staphylococci resistant to the penicillinase resistant penicillins, whether naturally occurring or made resistant *in vitro*, have by most authors been found not to cause any inactivation of these penicillins *Stewart & Holt* (21) however, isolated from clinical sources strains of *Staphylococcus aureus* resistant to the newer penicillins, which during growth inactivated isoxazolyl penicillins to a significant degree *Eriksen & Erichsen* (7 8) showed that also methicillin was inactivated in grow

Semisynthetic penicillins were kindly supplied by *H Lundbeck & Co A/S* cephalothin by *Eli Lilly and Co*

ing cultures of naturally occurring resistant strains These findings were confirmed by *Ayliffe & Barber* (3) and *Knox & Smith* (14), who also demonstrated a significant inactivation of methicillin and cloxacillin in full grown cultures of highly penicillinase-producing strains of *Staphylococcus aureus* induced with methicillin or cloxacillin, irrespective of sensitivity to these penicillins

The purpose of the present paper is to give a report of our continued studies on inactivation of methicillin caused by staphylococcal penicillinase under various experimental conditions, and of parallel studies with oxacillin, cloxacillin, and cephalothin

MATERIAL AND METHODS

The strains of *Staphylococcus aureus* used in these studies were as in previously reported experiments (8) S 5974 a naturally occurring methicillin resistant strain 2719 a methicillin sensitive highly penicillinase producing strain and 326 a penicillin sensitive strain The methicillin sensitive highly penicillinase producing staphylococcus 1634 which readily splits off penicillinase-negative mutants has been used in a few experiments

Tests for inactivation of methicillin oxacillin cloxacillin and cephalothin were carried out in actively growing cultures as well as in full grown cultures The medium used was nutrient broth ph 7 6 All experiments were carried out at 37° C.

Inactivation during active growth was tested in 5 ml cultures In most experiments a number of separate cultures was used In such cases all cultures were pipetted off from one bottle of medium to which drug and inoculum had just been added The inoculum was in all experiments an overnight broth culture which was diluted with broth in the proportion 1 10 giving an initial number of staphylococci of 100 150 × 10⁶ per ml

Full grown cultures were prepared in *Erlenmeyer* flasks with 50–100 ml of broth seeded with a middle sized inoculum After incubation for 18 hours the antibiotic contained in a volume of 0 5–1 ml was added and aliquots of 5 ml were distributed in tubes to be used for testing inactivation

Methicillin induced cultures were also large cultures grown in flasks Induction was carried out in two different ways In method 1 the culture was grown in broth containing 0 25 µg/ml of methicillin In method 2 methicillin was added to the 18 hour broth culture to give a concentration of 0 25 µg/ml The culture was then shaken for 6 hours at 37° C In both cases the induced cultures were tested for inactivating properties exactly as described above antibiotic being added and 5 ml aliquots being pipetted off

At specified intervals a test tube was removed from the incubator and the culture fluid was immediately passed through a *Seit* filter The filtrate which was sterile and entirely free of penicillinase (9) could be kept for several hours at room temperature without any further loss of antibiotic activity If stored for more than a few hours until testing for antibiotic activity could be carried out the filtrates were kept at 4° C

Residual drug activity was estimated by the agar cup method described by *Vesterdal* (22) The activity was calculated from dose response curves constructed from diameters of inhibition zones given by freshly prepared standard solutions of the particular penicillin

The test organism was for the assay of oxacillin cloxacillin and cephalothin *Staphylococcus aureus* 209 P For the assay of methicillin a strain of *B subtilis* was used

In each experiment a tube with uninoculated broth containing the same concentration of drug as used in the cultures was incubated At the end of the experiment also the control broth was *Seitz* filtered and assayed for drug activity No loss of activity was observed in any experiment described in this paper

RESULTS

Inactivation of methicillin during growth of methicillin resistant strains of *Staphylococcus aureus* has been tested in several experiments and with a number of different strains It was found that in broth with an initial concentration of 10 µg ml the activity after 24 hours incubation at 37° C was usually so small that no inhibition zone was seen when using staphylococcus 209 P as test organism The results of an experiment with staphylococcus S 5974 in which the activity was tested every two hours are shown in Table 1 A gradual decline in activity was observed beginning after 6–8 hours incubation

However, using cup plates seeded with staphylococcus 209 P concentrations of methicillin lower than 2 5 µg ml do not give any inhibition zones Therefore, in later experiments a strain of *B subtilis*, which is much more sensitive to the action of methicillin, has been used

It was subsequently observed that inactivation in cultures of S 5974 sometimes proceeded at a significantly slower rate, possibly due to the fact that the culture was not very stable in regard to penicillinase-production colonies of penicillinase-negative mutant strains often being found in significant numbers after spreading on agar plates (8) Therefore, a penicillinase producing strain was isolated from one single colony In all later experiments this strain, which proved very stable, was used It inactivated methicillin at a somewhat higher rate than did the strain used in the experiments shown in Table 1

TABLE 1
Inactivation of Methicillin during Growth of the Methicillin Resistant Staphylococcus aureus *S 5974 Remaining Activity Tested after 6 22 Hours. Initial Concentration of Methicillin 10 µg/ml*

Hours	Diameter of inhibition zone in mm
0	29
6	29
8	27
10	25
12	22
14	21
16	19
18	17
20	14
22	<10

In Table 2 is shown the inactivation which took place during gro th of th

... out with the methicillin sensitive penicillinase producing staphylococcus 2719 For all compounds a significant inactivation was observed during growth of the resistant strain

The sensitive strain caused inactivation of oxacillin and cloxacillin at a somewhat slower rate than did the resistant one. Also cephalothin was inactivated during growth of the sensitive strain. With the large inoculum used growth was obtained in all three compounds after 24–48 hours. In methicillin containing broth the sensitive strain did not grow and in this case inactivation proceeded at a very slow rate.

TABLE 2

Inactivation of Methicillin, Oxacillin, Cloxacillin and Cephalothin during Growth of a Methicillin-Resistant (S 5974) and a Methicillin-Sensitive (2719) Penicillinase Producing Staphylococcus aureus
Initial Concentration of Each Antibiotic 10 µg/ml

Hours	Methicillin		Oxacillin		Cloxacillin		Cephalothin	
	Remaining activity in µg/ml after incubation for 6–72 hours							
	S 5974	2719	S 5974	2719	S 5974	2719	S 5974	2719
6	–	–	3.4	5.0	2.8	4.4	10.0	10.0
8		–	0.6	3.6	1.7	3.6	6.4	8.0
10	–	–	<0.3	1.9	0.4	2.1		–
12	–	–	–	0.9	<0.3	1.8	2.5	8.0
24	0.7	8.0		–	–	<0.3	<0.3	2.0
48	<0.3	8.0		–			–	<0.3
72	–	5.0			–		–	

TABLE 3

Inactivation of Methicillin, Oxacillin and Cephalothin added to Non Induced Broth Cultures of a Methicillin Resistant (S 5974) and a Methicillin-Sensitive (2719) Penicillinase Producing Staphylococcus aureus
Initial Concentration of Each Antibiotic 10 µg/ml

Hours	Methicillin		Oxacillin		Cephalothin	
	Remaining activity in µg/ml after 12–120 hours					
	S 5974	2719	S 5974	2719	S 5974	2719
12	–		10.0	10.0	10.0	10.0
24	10.0	8.0	6.5	6.5	7.5	7.5
48	5.0	3.8	0.5	<0.3	1.3	0.9
72	3.2	2.5	<0.3		<0.3	<0.3
96	1.6	1.2		–		
120	0.8	0.8	–		–	

Table 3 shows the results of experiments in which the compounds were added to full grown cultures. For all three compounds tested inactivation took place at a very slow rate. The inactivating properties of the two different cultures were virtually identical.

The results of experiments with methicillin induced cultures are presented in Table 4–6. All four compounds were inactivated to a significant degree by induced cultures of both the methicillin-resistant and the methicillin sensitive staphylococcus, the cultures induced by method 2 being most active.

TABLE 4

Inactivation of Methicillin, Oxacillin, Cloxacillin and Cephalothin Added to Methicillin Induced Broth Cultures of a Methicillin Resistant (S.5974) and a Methicillin Sensitive (2719) Penicillinase Producing Staphylococcus aureus. *Induction by Growth in Methicillin Containing Broth (Method 1). Initial Concentration of Each Antibiotic 10 µg/ml*

Hours	Methicillin S.5974	Methicillin 2719	Oxacillin S.5974	Oxacillin 2719	Cloxacillin S.5974	Cloxacillin 2719	Cephalothin S.5974	Cephalothin 2719
	Remaining activity in µg/ml after ½–72 hours							
½		–	2.5	4.4	–	–	–	–
1	–	–	1.2	2.8	–	–		–
2	–	–	<0.3	0.9	2.1	4.0	10.0	10.0
3	–	–	–	0.4	0.9	2.4	7.0	8.5
4	–	–		<0.3	<0.3	1.3	4.6	5.0
6		–	–	–	–	<0.3	<0.3	4.2
8		–	–	–	–		–	1.0
12	–		–		–	–	–	<0.3
24	3.2	3.2	–	–	–	–	–	–
48	1.0	1.0		–	–		–	–
72	<0.3	<0.3			–	–	–	–

TABLE 5

(Method 2)
Initial Concentration of Each Antibiotic 10 µg/ml

Hours	Methicillin S.5974	Methicillin 2719	Oxacillin S.5974	Oxacillin 2719	Cloxacillin S.5974	Cloxacillin 2719	Cephalothin S.5974	Cephalothin 2719
	Remaining activity in µg/ml after ½–24 hours							
½			1.6	1.0	4.0	1.8		
1			0.4	<0.3	0.7	<0.3	6.0	4.2
2		–	<0.3		<0.3	–	3.6	1.8
4							<0.3	<0.3
8	2.2	1.8	–		–			
12	1.4	0.7			–	–	–	
24	<0.3	<0.3		–	–	–	–	–

Oxacillin and cloxacillin were inactivated at a particularly high rate. With an initial concentration of 10 µg/ml inactivation was virtually complete after 1–2 hours, with 100 µg/ml after 2–4 hours.

The slight differences observed between the inactivating properties of cultures of the methicillin-resistant and the methicillin-sensitive strain, respectively, were probably not significant.

In Table 7 are shown the results of an experiment in which the susceptibility to methicillin and cloxacillin of two penicillinase producing strains of *Staphylococcus aureus* has been compared ...

The sensitive strain caused inactivation of oxacillin and cloxacillin at a somewhat slower rate than did the resistant one Also cephalothin was inactivated during growth of the sensitive strain With the large inoculum used growth was obtained in all three compounds after 24–48 hours In methicillin-containing broth the sensitive strain did not grow and in this case inactivation proceeded at a very slow rate

TABLE 2

Inactivation of Methicillin Oxacillin Cloxacillin and Cephalothin during Growth of a Methicillin Resistant (S 5974) and a Methicillin Sensitive (2719) Penicillinase Producing Staphylococcus aureus
Initial Concentration of Each Antibiotic 10 µg/ml

Remaining activity in µg ml after incubation for 6-72 hours

Hours	Methicillin		Oxacillin		Cloxacillin		Cephalothin	
	S 5974	2719	S 5974	2719	S 5974	2719	S 5974	2719
6	–	–	3 4	5 0	2 8	4 4	10 0	10 0
8	–	–	0 6	3 6	1 7	3 6	6 4	8 0
10	–	–	<0 3	1 9	0 4	2 1	–	–
12	–	–	–	0 9	<0 3	1 8	2 5	8 0
24	0 7	8 0	–			<0 3	<0 3	2 0
48	<0 3	8 0	–	–	–	–	–	<0 3
72	–	5 0	–	–	–	–	–	–

TABLE 3

Inactivation of Methicillin Oxacillin and Cephalothin added to Non Induced Broth Cultures of a Methicillin Resistant (S 5974) and a Methicillin Sensitive (2719) Penicillinase Producing Staphylococcus aureus
Initial Concentration of Each Antibiotic 10 µg/ml

Remaining activity in µg ml after 12 120 hours

Hours	Methicillin		Oxacillin		Cephalothin	
	S 5974	2719	S 5974	2719	S 5974	2719
12		–	10 0	10 0	10 0	10 0
24	10 0	8 0	6 5	6 5	7 5	7 5
48	5 0	3 8	0 5	<0 3	1 3	0 9
72	3 2	2 5	<0 3	–	<0 3	<0 3
96	1 6	1 2			–	
120	0 8	0 8				

Table 3 shows the results of experiments in which the compounds were added to full grown cultures For all three compounds tested inactivation took place at a very slow rate The inactivating properties of the two different cultures were virtually identical

The results of experiments with methicillin induced cultures are presented in Table 4 6 All four compounds were inactivated to a significant degree by induced cultures of both the methicillin resistant and the methicillin-sensitive staphylococcus, the cultures induced by method 2 being most active

DISCUSSION

The possible significance of inactivation by penicillinase for anti staphylococcal activity and clinical effect of the so called penicillinase-resistant penicillins is a matter in dispute

Reports demonstrating that both methicillin and isoxazolyl penicil lins are inactivated by penicillinase from *B cereus* and when they are present in high concentrations also by staphylococcal penicillinase (15 16 18 20) did not cause any change in the commonly held view that these penicillins for all practical purposes are unaffected by penicil linase Despite amply confirmed reports that isoxazolyl penicillins are less penicillinase-resistant than methicillin (15 18) some authors even regard all these penicillins as equally resistant to penicillinase (2)

The current view concerning penicillinase resistance of the new penicillins however must be fundamentally changed as a consequence of the recent findings that both methicillin and isoxazolyl penicillins are inactivated during growth of methicillin resistant staphylococci and that cultures of penicillinase-producing staphylococci whether methi cillin resistant or not inactivate these penicillins when pre induced with methicillin or cloxacillin (3 7 8 14 21)

The experiments reported in the present paper have fully confirmed that methicillin oxacillin and cloxacillin under certain experimental conditions are inactivated by staphylococcal penicillinase also when present only in therapeutic concentrations Also the semi synthetic cephalosporin cephalothin was inactivated at a significant rate

The rate of inactivation particularly of the isoxazolyl penicillins was in some of our experiments so considerable that it appears highly prob able that inactivation is a factor of importance for the action of these penicillins both *in vitro* and *in vivo*

Experimental results published in a previous paper (8) indicated that penicillinase activity contributes significantly to the resistance of naturally occurring methicillin resistant strains of *Staphylococcus aureus* However methicillin resistance is not only a result of penicil linase activity and at least some methicillin resistant strains of *Staphy lococcus albus* isolated from clinical sources do not inactivate methicil lin at all as shown by *Kjellander et al* (11 12) We have examined a few highly methicillin resistant strains and have found that even methi cillin induced cultures of such strains do not inactivate methicillin

Also for the *in vitro* activity against methicillin sensitive staphylo cocci however penicillinase-activity appears to be of some significance

Thus the rather marked inoculum size effect observed with penicil linase producing staphylococci against both isoxazolyl penicillins and cephalosporins has been regarded as being due to inactivation by peni cillinase (4 8)

Sabath & Finland (17) made a thourough study of the stability of methicillin and oxacillin in fluid medium inoculated with penicillinase

of the two penicillinase-producing strains was significantly lower than that of the non-penicillinase-producing strains. Also against methicillin the penicillinase-producing strains were somewhat less sensitive than the penicillinase-negative strains. The difference, however, was only evident when the figures relating to confluent growth were compared. In the case of oxacillin the difference in activity was approximately the same whether estimated from the observation of confluent growth or of complete growth inhibition

TABLE 6

Inactivation of Methicillin, Oxacillin, Cloxacillin and Cephalothin Added to Methicillin-Induced Broth Cultures of a Methicillin Resistant (S 5974) and a Methicillin-Sensitive (2719) Penicillinase-Producing Staphylococcus aureus *Induction by Shaking Broth Cultures for 6 Hours after Addition of Methicillin (Method 2).*
Initial Concentration of Each Antibiotic 100 µg/ml

Hours	Remaining activity in µg ml after ½–48 hours							
	Methicillin		Oxacillin		Cloxacillin		Cephalothin	
	S 5974	2719	S 5974	2719	S 5974	2719	S 5974	2719
½	–	–	32	22	–	–	–	–
1	–	–	5.0	0.4	25	8.0	–	–
2	–	–	0.6	<0.3	9.0	0.9	–	–
3	–	–	<0.3	–	2.3	<0.3	–	–
4	–	–	–	–	<0.3	–	–	–
8	25	25	–	–	–	–	70	70
12	18	16	–	–	–	–	60	50
24	1.6	1.6	–	–	–	–	<0.3	<0.3
48	<0.3	<0.3	–	–		–	–	–

TABLE 7

Sensitivity to Methicillin and Oxacillin of Penicillinase-Producing and Non-Penicillinase-Producing Strains of Staphylococcus aureus, *Estimated on Solid Medium Inoculated with 0.1 ml Undiluted Broth Culture (100–150 × 10⁶ Bacteria)*

	Methicillin	*Oxacillin*
Penicillinase-producing *Staphylococcus aureus* 2719	1.25 (1.25)	0.312 (1.25)
Penicillinase producing *Staphylococcus aureus* 1634	1.25 (1.25)	0.312 (0.625)
Penicillinase-negative strain derived from *Staphylococcus aureus* 1634	0.625 (1.25)	0.039 (0.156)
Penicillinase-sensitive *Staphylococcus aureus* 326	0.625 (1.25)	0.039 (0.078)

For each strain the first figure gives the highest concentration showing confluent growth. The figure in brackets shows the highest concentration in which growth occurred at all.

ly inactivated by staphylococcal penicillinase which is produced in large amounts also in non induced cultures

The penicillinase-resistant penicillins are much more active inducers of penicillinase than is penicillin G a property presumably related to their penicillinase resistance (15 16 18 20) It appears probable that these penicillins under certain conditions will be active inducers also *in vivo*

As optimal induction of staphylococcal penicillinase occurs only when the bacteria are actively multiplying (19) it is less likely that induction will take place if the staphylococcus is methicillin sensitive because growth of the bacterial cells will be inhibited by the antibiotic If tissue concentration of antibiotic is very low which may happen for various reasons of course induction may be supposed to take place also when the staphylococci are methicillin sensitive

Ayliffe & Barber (3) and *Knox & Smith* (14) have emphasized that inactivation of methicillin and cloxacillin even in induced cultures is infinitesimal as compared with the inactivation of penicillin G But this fact does not necessarily mean that inactivation has no practical implications Inactivation may very well be a significant factor interfering with the therapeutic effect of penicillinase resistant penicillins and cephalothin in infections with methicillin resistant staphylococci and perhaps if induction is allowed to take place during an initial phase with very low tissue concentrations also in infections caused by methicillin sensitive staphylococci

SUMMARY

Inactivation of some penicillinase resistant penicillins and cephalothin by staphylococcal penicillinase has been tested under various experimental conditions During growth of a methicillin resistant strain of *Staphylococcus aureus* a significant inactivation was observed The isoxazolyl penicillins were inactivated also during growth of a meth

...sults of some comparative experiments with penicillinase producing and non penicillinase producing strains of *Staphylococcus aureus* indicate that penicillinase activity contributes to the *in vitro* resistance of penicillinase producing staphylococci against both oxacillin and methicillin

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producing staphylococci They observed a secondary outgrowth of staphylococci with unchanged sensitivity to methicillin and oxacillin coincident with a reduction of antibiotic concentration to subinhibitory values

Klein et al (13) suggested that the destruction of oxacillin accounts for differences in sensitivity observed between penicillinase-producing and penicillin-sensitive strains of *Staphylococcus aureus*

Abu-Nassar et al (1), however, found no relationship between penicillinase production and the resistance of staphylococci to methicillin and oxacillin

Experiments presented in this paper (Table 7) have demonstrated a considerable difference in sensitivity to oxacillin between penicillinase-producing and non-penicillinase-producing staphylococci Also against methicillin are the penicillinase-producers more resistant than the penicillinase-negative strains

Particularly noteworthy is the difference in sensitivity between the penicillinase-producing staphylococcus 1634 and the penicillinase negative strain derived from it This mutant strain was used also in previously published experiments and was shown to be fully sensitive to penicillin G (8) It appears from Table 7 that its susceptibility also against oxacillin and methicillin is the same as that of an ordinary penicillin-sensitive staphylococcus The experimental results obtained with this strain appears to carry a strong evidence in favour of the hypothesis that penicillinase activity contributes to the *in vitro* resistance of penicillinase-producing staphylococci against both oxacillin and methicillin

Sabath & Finland (17) have suggested that inactivation may account for difficulties observed in some cases in eradicating staphylococci during methicillin therapy Persistence of staphylococci during treatment with the new penicillins has been observed also by other authors (5)

Inactivation of methicillin in uninduced cultures of a methicillin-sensitive staphylococcus as observed by *Sabath & Finland* and as demonstrated also in the present paper (Table 2) proceeds at a very slow rate Thus, judging from experiments of this kind, it does not seem probable that inactivation of methicillin in the presence of methicillin-sensitive staphylococci can be a factor interfering with successful therapy

If, however, induction of penicillinase formation takes place *in vivo* inactivation might be supposed to be a significant factor, in view of the high rate at which methicillin-induced cultures inactivated all compounds tested in the present investigation

Several authors have suggested that induced formation of staphylococcal penicillinase may take place *in vivo* during treatment with penicillin G, but its occurrence has never been proved (6, 10) Possibly, induction it not very important because penicillin G is extremely rapid-

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The Department of Bacteriology (Head Professor Gunnar Lofstrom),
University of Uppsala Sweden

THE ISOLATION OF SALMONELLA IN A SWEDISH WATER COURSE (THE RIVER FYRIS)

1 *Isolation by Various Filter Methods and the Swab Technique According to Moore*[1]

By

ABERRA DEMISSIE

Received 20 ix 64

In isolating enteropathogenic salmonella from different kinds of water, such as tap water, well water and river water, different methods have come into use

The committee on Bathing Beach Contamination (1959) obtained positive results by filtering large volumes of sea water through Sterimat filter of porosity FCB and by culturing the pads

Collet and her collaborators (1953) have successfully isolated *S typhi* from a well located near the home of a typhoid carrier by filtering varying quantities of water through membrane filter

Ljutov (1954) employed Hyphlo supercel (the fossile remains of diatorns) and a textile fabric in demontrating salmonella bacteria in water by filtering large quantities

A simple technique for the location of enteropathogenic bacteria in towns by means of serial sewage examinations was described by *Moore* (1948, 1950)

In the comparative studies presented here, the above filters and a modified Moore's swab were used to isolate enteropathogenic salmonella from a swedish watercourse (the river Fyris) during a four months period (May–August 1963)

MATERIAL AND METHODS

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[1] This investigation was made possible through a scholarship grant from NIB (*Nämnden för internationellt bistånd*) and financial assistance from the *Department of Bacteriology*

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A simple technique for the location of enteropathogenic bacteria in towns by means of serial sewage examinations was described by *Moore* (1948 1950)

In the comparative studies presented here the above filters and a modified Moore s swab were used to isolate enteropathogenic salmonella from a swedish watercourse (the river Fyris) during a four months period (May–August 1963)

MATERIAL AND METHODS

A *The Water Course in Question and Plan of Investigation*

The River Fyris originated from a small lake in the North of Uppland collected water from smaller streams passed through Uppsala and fell into lake Mälaren

Three sites Jarnbron Harbour and Flottsund referred to as I II and III respectively hereafter were chosen for the present work Site I was located in the upper

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E *Experimental*

Preliminary tests with water artificially infected with S typhi murium were designed to evaluate the sensitivity of the filtration methods The test organism *S typhi murium* had been isolated from the river The bacteria were grown over night at 37° C and diluted to 10⁻⁷ 10⁻⁸ and 10⁻⁹ in saline The viable count was determined by plating 0 3 ml of each dilution on a large Endo plate One ml of each dilution was added to three litres of river water The water used for the 10⁻⁹ dilution had been sterilized but non sterilized water was used for the two other dilutions 10 experiments were undertaken for each dilution

RESULTS

The General Character of the Various Filter Methods

In using the membrane filter in filtering polluted river water difficulties were encountered It became evident that the membrane filter was too slow, requiring 4–5 hours to filter a three-litre-sample of specimen due to the blocking effect of particles in the impure river water

The use of this filter, was therefore abandoned at an early stage of the investigation

It is seen from Table 1 that about 13 per cent of the organisms present in the original sample passed through when 0 5 gr of supercel was used, compared to about 6 per cent when the supercel material was raised to 1 0 gr The larger amount of filter material made the filtration more difficult as in the case of the membrane filter Three litres of river water was filtered in about ½ hour and 10 litres in about three hours

TABLE 1

MPN Test on the Number of Coliform Bacteria Passing through Supercel- and Seitz Filters

No of experiments	Filter method	Coliform 100 ml		Plate count 37° C
		37°C	44°C	
9	Untreated	8444 ± 950 8	1789 ± 847 6	1528 ± 896 1
9	0 5 gr supercel	1078 ± 777 8	478 ± 705 7	178 ± 87 6
9	1 0 gr supercel	522 ± 440 5	144 ± 389 9	122 ± 93 2
9	Seitz	–	–	–

The results obtained by the Seitz filter were found to be similar to those obtained by the supercel method Filtration of large quantities (9) took close to two hours While some bacteria escaped through the Seitz filter (Table 1)

Preliminary tests in which the supercel and the Seitz filter were used in combination, first filtering through supercel (0 5 gr) and then through Seitz filter, showed no passing through of the bacteria The time required to filter 9 litres of raw river water was 2 hours

The Isolation of Salmonella Bacteria

The isolation of Salmonella is demonstrated in Table 2 20 specimens of water were filtered through supercel filter and *S typhimurium* was

found in one specimen 5 filtrations through Seitz filter were negative 61 swabs were examined and three salmonella strains were isolated from the river and five from the manholes

TABLE 2
The Isolation of Salmonella Bacteria in Water From the River Fyris and in Sewagewater Manholes of the Sewage System of Uppsala

Technique for isolation	Source	No of specimens	Pos no of speci mens	Date of isolation	Types of strains isolated
Swab	Harbour	40	1	10 6	*S newport*
			1	25 6	*S paratyphi B*
			1	29 7	*S blockley*
Swab	Manhole	21	3	27 8	*S blockley*
				2 9	*S blockley*
				10 9	*S blockley*
			1	18 9	*S typhimurium*
			1	10 9	*S enteritidis*
Supercel	Harbour	20	1	25 6	*S typhimurium*
Seitz	Harbour	5	0		—

TABLE 3
An Epidemiological Study of a Case of S blockley by Isolation of Bacteria from Stool a Manhole of the Sewage System and Water of the River Fyris at the Outlet from the Sewage Treatment Plant

Date	Isolation of S Blockley		
	Faeces	Manhole	River water
13 5	pos +		
5 6	neg —		neg —
17 6	pos +		neg —
29 6	pos +		neg —
29 7	pos +		neg —
25 8	pos +	pos +	pos +
3 9	neg —	pos +	
12 9	neg —		
16 9	neg —	pos +	
20 9	neg —	neg —	

Epidemiological Survey

One of the salmonella strains that was isolated from the river, at site II was *S blockley* as shown in the Table 2 Only one case caused by this type of salmonella was known in the town

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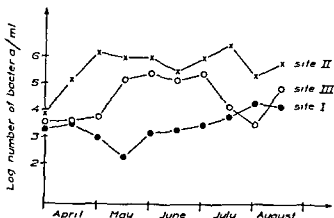


Fig 2
Number of coliform in the river Lyris during the spring and summer 1963

tween June 17 and August 25 She was thus a healthy carrier and was readmitted for treatment to the hospital on August 28 Faeces became negative on September 3, and remained negative in four specimens, the last one examined on September 20

Salmonella Blockley was found in water from site II (see Fig 1) on July 29, which finding started the examinations of the manholes Thus *S blockley* was isolated from a manhole adjacent to the house in which this patient lived on August 25, September 3, and 16 An examination on September 20, was negative

The same manhole was positive for *S enteritidis* as well as *S typhimurium* of the same phage type as the *S typhimurium*, which had been isolated from the river almost three months earlier The strains could not be related to any known carrier

The additional strains which had been isolated from the river, were *S newport* and *S paratyphi B* They were not found in the sewage system No carrier of *S newport* was known in the town An extensive epidemic caused by this strain, however, had occurred one year earlier A patient with *S paratyphi* B infection had been living in the town shortly before the time at which the strain was isolated from the river

The coliform test The results of the MPN estimation of the coliform density are presented in Fig 2 As may be seen from the figure, there was variation in the number of bacteria from the time the test began on April 17 to the time the work was concluded on August 27, 1963 The number of coliform bacteria increased slightly the first fortnight in specimens from I and III but II showed a 20 fold increase The increase was from 2000 coliform/100 ml, 8000 coliform/100 ml and 3000 coliform/100 ml to 3000 coliform/100 ml, 150 000 coliform 100 ml and 4000 coliform/100 ml for I, II and III respectively The peak of the coliform density for II and III was reached during the summer with 1 500 000 coliform/100 ml and 50 000 coliform/100 ml respectively The coliform content of site I showed less variations

TABLE 4

Dilution Experiment with S typhimurium in Water from the River Fyris Using the Supercel Technique and Seitz Filter

No of experiments	Dilution	No of pathogen bact per ml	Volume of water ml	No of positive experiments		
				Supercel filters		Seitz filter
				0 5 gr	1 0 gr	
10	10	159	3000	10	10	10
10	10^{-8}	26	3000	8	10	10
10	10^{-9}	<1	3000	0	3	5

Dilutions Experiments

The results of the dilution experiments are presented in Table 4 In dilution 10^{-7} containing about 159 *S typhimurium* per ml it was possible to recover the test organism irrespective of the amount of supercel whether 0 5 or 1 0 gr was used The test organism was also recovered by the Seitz filter The result with the 10^{-8} dilution however, was somewhat different The test organism was recovered in all 10 trials when 1 0 gr of supercel was used but only in 8 trials with 0 5 gr of supercel The seitz filter gave positive results in all 10 tests

The water sample was sterilized before adding the test bacteria in a dilution of 10^{-9} This dilution gave no growth of bacteria on plates Calculated from the lower dilutions the number of bacteria should have been 1 3 bacteria per ml *S typhimurium* was recovered 3 times when 1 0 gr of supercel was used against none when the weight of this material was lowered to 0 5 gr The Seitz filter recovered the bacteria in one half of the experiments

DISCUSSION

The use of the swab according to *Moore* had proven itself as an effective tool in epidemiological surveys on river and sewage water Salmonella bacteria could be isolated in the river Fyris and in the related sewage system On one occasion an infection of *Salmonella Blockley* was the only case known in the town Bacteria were isolated simultaneously from faeces of the case the sewage system and the river The strain remained in the sewage system for about a fortnight longer than it could be isolated from the patient The ordinary swab technique did not give quantitative results which might be a weakness in some instances There is a possibility however utilized by *Moore* to get such results by passing the water through a device measuring the volume of passing water

In filtration tests on larger volumes of polluted water the result was influenced by the size of the pores There was naturally enough an inverse relationship between the filtration time and the capacity of the filter to retain pathogenic bacteria Due to these circumstances the

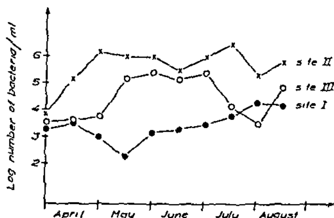


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Virus Dept B National Institute of Public Health Oslo and Dept of Microbiology
Dental Faculty University of Oslo

LACK OF CHICK ALLANTOIC ANTIGEN IN INFLUENZA VIRUS PASSED IN CHICK KIDNEY MONOLAYERS

By

G ROLLA

Received 25 iv 64

The influenza virus haemagglutination inhibiting antibody produced after injection of normal host material, was first reported by *Knight*, and further investigated by *Harboe* (6, 1, 2, 3, 4) *Strandli, Mortensson-Egnund & Harboe* have recently published a method for the further purification of this antigen from normal chick allantoic fluid (8) A chemical analysis of the antigen showed it to be an acid mucopolysaccharide

The experiments described in the present report intend to find out whether influenza virus propagated in chick cell monolayers is inhibited by antiserum against chick allantoic material This antiserum inhibits viruses grown in the allantoic sac of chick embryos

In an additional experiment influenza virus grown in duck embryos was treated with normal chick allantoic fluid and subsequently tested for the presence of normal chick allantoic antigen

MATERIALS AND METHODS

Virus The influenza strains A/PR8 and B/Lee were used The strains were grown in the chick embryo duck embryo or chick kidney cell monolayers They were purified by adsorption to and subsen ent el t on fr m f
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1 after inoculation of virus
nedium was changed every
with virus the monolayers
virus suspension was then
au oi t on for 90 minutes at 37° C The
ure was washed three times in Hanks solution and medium was then added
After four to five days the tubes were tested f r th
Haem

supercel filter using 0 5–1 gr of the material was the most suitable of the filters tested in epidemiological work on Salmonella The supercel filter also showed its *good quality in the dilution experiments with S typhimurium* The pore-size of the steremat- and Seitz filters was too small The bacteria were retained, but the filters were blocked by the impurities of the water, and the filtration took considerable time There was a feasible prospect of using the supercel- and Seitz filters in combination

The sewage system of Uppsala included a sewage treatment plant, which could apparently be passed by the Salmonella bacteria The reason was not known It could be seen that the number of coliform bacteria in the river varied considerably *from* time to time The peak occurred during the summer months when the flow of water of the river had slowed down Salmonella bacteria were found, in the river water during the whole summer

SUMMARY

In a four months period, May-August 1963, a survey was conducted as to the occurrence of salmonella in the water of Fyrisån Different methods were tested The results of a modified swab technique according to *Moore* and of filtration through Membrane (MF), supercel and Seitz-filter was compared The following strains of salmonella were isolated *S newport, S typhimurium,* phage type NS, *S paratyphi B,* phage type 3 AI var 2, *S blockley* and *S enteritidis* The modified swab technique gave the highest number of positive results One of the strains, *S blockley,* was traced back to a manhole located in the vicinity of the only patient, who was known to be carrier of this strain in Uppsala at that time

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Virus Dept B National Institute of Public Health, Oslo and Dept of Microbiology, Dental Faculty, University of Oslo

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By

G RÖLLA

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The influenza virus *haemagglutination-inhibiting antibody produced* after injection of normal host material, was first reported by *Knight*, and further investigated by *Harboe* (6, 1, 2, 3, 4) *Strandli, Mortensson-Egnund & Harboe* have recently published a method for the further purification of this antigen from normal chick allantoic fluid (8) A chemical analysis of the antigen showed it to be an acid mucopolysaccharide

The experiments described in the present report intend to find out whether influenza virus propagated in chick cell monolayers is inhibited by antiserum against chick allantoic material This antiserum inhibits viruses grown in the allantoic sac of chick embryos

In an additional experiment influenza virus grown in duck embryos was treated with normal chick allantoic fluid and subsequently tested for the presence of normal chick allantoic antigen

MATERIALS AND METHODS

Virus The influenza strains A/PR8 and B/Lee were used The strains were grown in

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mycin pr ml The same medium was used after inoculation of virus except it contained only 2 per cent calf serum The medium was changed every 24 hours during the growth period Before inoculation with virus the monolayers were washed three times with Hanks solution 0 1 ml of virus suspension was then added to each tube which was left for adsorbtion for 90 minutes at 37° C The culture was washed three times in Hanks solution and

After four

Haemagg

according

was treated with five volumes of cholera filtrate (Philips Duphar) to destroy normal inhibitors (9) Serum haemagglutinin was adsorbed with 10 per cent fowl red cells

RESULTS

1 *Influenza Virus Passed in Chick Kidney Tissue Culture*

A/PR8 was passed 4 times and B Lee 5 times in chick kidney cells The red cell eluates of the viruses were tested with rabbit antiserum against host material No inhibition could be demonstrated The results are given in Table 1

TABLE 1

Haemagglutination Inhibition Test Showing The Absence of Normal Chick Allantoic Antigen in Tissue Culture Grown Viruses

Virus antigen	Tested with antiserum to B Lee	Tested with antiserum to A PR8
A/PR8 from the chick allantoic sac	48	2000
B/Lee from the chick allantoic sac	3000	96
A/PR8 propagated in chick kidney cell monolayers	<6	1500
B/Lee propagated in chick kidney cell monolayers	2000	<6

TABLE 2

Haemagglutination Inhibition Tests for Normal Chick Allantoic Antigen in Purified Duck Embryo Grown Viruses Treated with Normal Chick Allantoic Fluid

Virus antigen	Tested with antiserum to B Lee	Tested with antiserum to A PR8
A/PR8 from the duck allantoic sac (9 passage)	<6	1500
B/Lee from the duck allantoic sac (8 passage)	2000	<6
Purified A/PR8 from the duck allantoic sac treated with normal chick allantoic fluid for		
2 hours at room temp	<6	1500
24 hours at 5° C	<6	1500
Purified B/Lee from the duck allantoic sac treated with normal chick allantoic fluid for		
2 hours at room temp	2000	<6
24 hours at 5° C	2000	<6

The table shows that antiserum to B Lee grown in the chick allantoic sac moderately inhibits allantoic A/PR8 and that antiserum to allantoic A/PR8 inhibits allantoic B/Lee This inhibition of unrelated virus is caused by the antibody to host material (6, 2, 3 4 5) It is further seen from the table that virus propagated in chick tissue cultures was not inhibited by antibody to chick allantoic material

2 *Influenza Virus Passed in Duck Embryos and Treated with Normal Chick Allantoic Fluid*

After several passages, red cell eluates were prepared and tested for presence of normal chick allantoic antigen As this test thus gave no

evidence for the presence of this antigen the purified duck embryo viruses were transferred to normal chick allantoic fluid and left for two hours at room temperature or *24 hours at 5° C The purification* procedures were then performed once more and the viruses tested for the presence of normal chick allantoic antigen The results are given in Table 2

The table shows that viruses cultured in the duck allantoic sac were not inhibited by antibody against chick allantoic fluid even when the viruses had been pretreated with chick *allantoic fluid*

DISCUSSION

It has been shown that the normal chick allantoic antigen is present in preparations of influenza virus from the chick allantoic sac but not in duck allantoic sac or mouse lung cultured virus (3) Cross reacting antigens have been demonstrated in duck and turkey normal allantoic fluids but not in chick embryo viscera or guinea pig kidney (4)

The first experiments described in this report show that no chick allantoic antigen demonstrable in the HI test, is present in the viruses propagated in chick kidney cells

In order to see if allantoic antigen material was attached to mature virus particles and thus might give an explanation to the phenomenon of the haemagglutination inhibition of antiserum to host material the *duck allantoic viruses were treated with chick allantoic fluid* The subsequent HI test showed however that no such attachment took place

SUMMARY

1 The haemagglutinin of influenza virus grown in chick embryo kidney tissue culture was not inhibited by antiserum against chick allantoic material

2 Antiserum to chick allantoic material did not inhibit the haemagglutinin of influenza virus grown in the duck allantoic sac and treated with normal chick allantoic fluid

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Acta path et microbiol scandinav 62 421–431, 1964

The Rickettsia and Virus Department, Statens Seruminstitut Copenhagen

THE INCIDENCE OF COMPLEMENT-FIXING ANTIBODIES TO THE RESPIRATORY SYNCYTIAL VIRUS IN SERA FROM DANISH POPULATION GROUPS AGED 0–19 YEARS

By

ALLAN HORNSLETH and MOGENS VOLKERT

Received 11 iv 64

Since its detection in 1956 (3, 4) as a cause of human respiratory disease the Respiratory Syncytial (RS) virus has been repeatedly and firmly established as an important aetiological factor in severe respiratory infections in infants and children in the United States (1, 2, 5, 11, 14) in Great Britain (9, 17, 18), and in Australia (13) In adults it only seems to give infections resembling the common cold (7, 10, 12)

Since in one major study (8) it was found to be the most important single aetiological factor in acute respiratory illness in children less than eight years of age, and in view of the high percentage of sera from different age groups in England found to contain antibodies to this virus 15), it seemed to be of value to obtain an estimation of the antibodies present in Danish population groups

MATERIAL AND METHODS

Blood samples collected from all over Denmark during a one year period blood samples from children with acute respiratory diseases admitted to hospitals in Copenhagen during the same period and a number of sera stored in this department during six consecutive years constitute the material

Sera examined a) 1324 single sera taken during the year August 1962 to July 1963 The number corresponds to an annual 57 68 patients at ages from 1 to 19 years plus 58 from the 0–5 months age group and 57 from the 6–11 months age group 661 of the sera were collected from August to December 1962 and 663 from February to July 1963

All the blood specimens were sent to the Treponematoses Department at the Statens Seruminstitut (for Wasserman testing) 79 were collected in general practice and the rest came from patients admitted to hospitals (both medical and surgical departments)

b 151 paired sera which had been tested in the Influenza Department at this

The authors are indebted to Dr *R M Chanock* [illegible]

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b) 151 paired sera which had been tested in the Influenza Department at this

The authors are indebted to Dr *R M Chanock* (Bethesda [illegible]

Institute for complement fixing antibodies against influenza and adenovirus during the period September 1962 to June 1963

c) 268 single sera examined in this department by means of the psittacosis complement fixation test during the years 1957 to 1962 The majority of these sera were from the Copenhagen area

All the specimens were heated for 30 minutes at 56° C and stored at —20° C until examined

Virus strain The Long strain of the RS virus, kindly provided by Dr *R M Chanock* National Institutes of Health, Bethesda, U S A , was used throughout

Tissue culture HeLa cells grown in Roux bottles in Hanks medium containing 10 per cent horse serum and 0 5 per cent lactalbumin One hundred units of penicillin and 100 micrograms of streptomycin per ml were added to all the media The maintenance medium used in the preparation of complement fixing antigen was the same as in the assay of virus titres, except that it contained only 5 per cent horse serum

Preparation of antigen Roux bottles seeded three days previously with seven million cells per bottle were inoculated with 8–10 ml of maintenance medium containing approximately 10^{3} TCD_{50} RS virus per ml When more than 90 per cent of the cells in the monolayer showed cytopathology (40–50 hours after inoculation), the bottles were frozen and thawed three times The harvested cells plus medium were centrifuged at 2000 r p m for 30 minutes and the supernatant fluid used as the antigen The antigen was measured by the usual box titration and comparison of different lots of antigen was always made on the same day against the same serum

Chanock et al (3) showed that ultracentrifugation of infectious fluid for several hours failed to diminish the potency of the soluble CF antigen in the supernatant fluid In our experiments it was found that ultracentrifugation for one hour (Spinco swinging bucket 39 1) at 30 000 r p m usually doubled the titre of the antigen present in the supernatant fluid after the preliminary low speed centrifugation This was always accompanied by a decrease in the anticomplementary activity of the antigen thus indicating that ultracentrifugation probably caused removal of anticomplementary factors By means of ultrafiltration (with a LKB filter type 6300 A) the titre of the antigen present in the ultraconcentrate was doubled when this fraction consisted of 15 20 per cent of the original volume, but no increase in titre was seen when the ultraconcentrate comprised more than 30 per cent of the original volume

The complement fixation test Serum dilutions two units of antigen and two units of complement each in amounts of 0 1 ml were mixed and kept overnight at 4° C for fixation After addition of the haemolytic system (two units of haemolysin 0 1 ml and a two per cent sheep erythrocyte suspension 0 1 ml) the tubes were placed in a water bath at 37° C for one hour Reading was made after further 30 minutes at room temperature The end point of the titration was read as the last tube with at least 50 per cent complement fixation Normal cell antigens and positive as well as negative sera were included as controls in all test series

EXPERIMENTAL

Incidence of Complement-Fixing Antibodies to the RS Virus in 1324 Sera Collected from August 1962 to July 1963

As can be seen from Table 1 the sera were collected from most parts of Denmark 661 of the sera were taken during the fall of 1962 and 663 sera during the spring of 1963 Moreover, the percentages concerning distribution according to locality were, broadly speaking, the same for the age-group 0–9 years as for the age-group 10–19 years The total number of sera containing complement fixing antibodies to the RS virus was 171 (13 per cent) From Table 1 it is apparent also that positive sera can be obtained from every part of the country without accumulation of these in any particular area

Fig 1 shows the distribution of positive sera in relation to age It is apparent that there is a steady increase in the numbers of positive reac-

TABLE 1

1324 Blood Specimens Examined for Complement Fixing Antibodies to the RS Virus

Place of origin	Numbers of sera examined	Numbers of CF positive sera
Copenhagen	450 (33 7)*	54 (12 0)†
Zealand (excl Copenhagen)	152 (11 5)	20 (13 1)
The islands of Lolland and Falster	34 (2 6)	2 (5 8)
The island of Funen	112 (8 5)	17 (15 2)
Northern Jutland (incl the town of Aalborg)	112 (8 5)	21 (18 7)
The town of Aarhus	306 (23 2)	37 (12 0)
Central part of Jutland (excl the town of Aarhus)	121 (9 2)	15 (12 4)
Southern part of Jutland	29 (2 2)	3 (10 3)
The island of Bornholm	8 (0 6)	2 (25 0)
Total	1324	171 (13 0)

* Per cent of total
† Per cent of the local number of sera

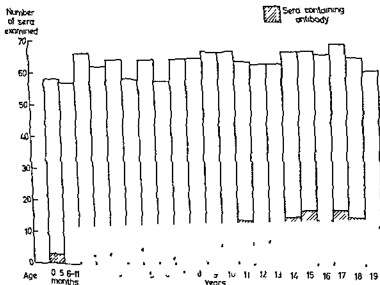


Fig 1

Distribution according to age of 1324 sera examined with the RS virus antigen in CF test during the period August 1962 to July 1963 The sera containing antibody are indicated

tions the mean percentages for the age-groups, 0–4, 5–9, 10 14, and 15–19 being 5 2, 8 3, 18 9 and 23 2, respectively (the 58 sera from patients 0–5 months of age are not included) When the total numbers of positive sera from the two age groups 0 9 years and 10–19 years (42 and 129 respectively) are compared the difference is found statistically significant (p less than 0 001 in the chi square test) When the sera are

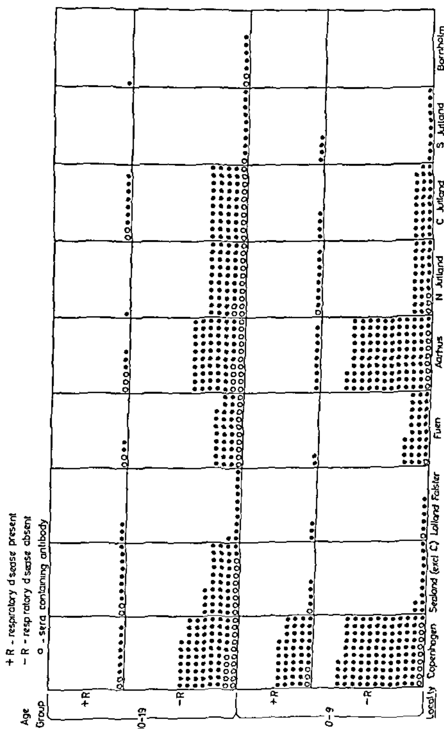


Fig 2

Distribution according to locality of sera from 705 patients without and 126 patients with acute respiratory disease at the time when the blood specimens were collected *The patients are divided into two age groups 0–9 and 10 19 years*

divided into two groups according to year of collection (August to December 1962 and February to July 1963) the same increase in the number of seropositives with age was seen for both these groups

A retrospective investigation of the 1324 sera involved was carried out With the kind co operation of hospitals and general practitioners it was possible to establish, with considerable certainty, from the case histories of 831 of the patients whether the patient had an acute re spiratory disease at the time when the blood specimen was taken Fig 2 shows the result of the study of these patients Statistically analysis of the data given in this scatter diagram indicates strongly that the pres ence of complement fixing antibodies to the RS virus had no relationship to the presence of acute respiratory diseases at the time the blood samples were taken neither in the younger nor in the older age groups Moreover in accordance with Table 1 this figure demonstrates the fairly even geographical distribution of antibodies to the RS virus in individuals in this country

The fact that the RS virus complement fixing antibodies are found so often in patients without acute respiratory diseases and the fact that the incidence of positive sera increases with age indicates strongly that these antibodies to the RS virus persist for many years in the blood of humans

Estimation of the RS Virus as an Aetiological Factor in Acute Respiratory Diseases in Children in Copenhagen in 1962–63

151 paired sera from infants and children aged 0 9 years were examined for the presence of complement fixing antibodies to the RS virus This group of sera originated from patients suffering from respiratory infections (Table 2) and represented most of the blood specimens sent to the Influenza Department from paediatric departments in Copen hagen hospitals from late September 1962 to the beginning of June 1963 The two specimens were taken at intervals of 5 to 13 days The age distribution of the 151 patients is given in Fig 3 which also shows the sera in which antibody to the RS virus was present As can be seen, the antibodies were found in one or both serum samples in only 20 of these patients

Table 3 shows the complement fixing antibody titres of the abovementioned 20 pairs of sera showing the presence of antibodies the corresponding age and clinical diagnosis of the patient together with the month in which the first blood sample was taken It can be seen that eight pairs of sera gave a more than two-fold rise in titres Moreover as many as seven of these were found among the 58 serum pairs taken in January February and March whereas only one belonged to the 93 serum pairs collected in the months before and after this period It is also apparent that all the patients who showed a significant rise in antibody titre were less than four years old and had pneumonic in

fections (Patient 13711 also had thrombocytopenic purpura and patient 13598 associated croup) None of the 20 patients had a CF antibody rise to influenza A, B or C, and only one (13115) had a CF antibody rise to adenovirus

TABLE 2

Distribution According to Main Diagnosis of 151 Infants and Children with Acute Respiratory Disease Admitted to Hospitals in Copenhagen from September 1962 to June 1963

Diagnosis	*Number of patients*
Pneumonitis	73
Bronchopneumonitis	17
Acute laryngitis	8
Primary atypical pneumonitis	9
Viral pneumonitis	6
Acute bronchitis	*22*
Pneumonic infiltration	6
Acute rhinopharyngitis	6
Catarrhalia	4
Total	151

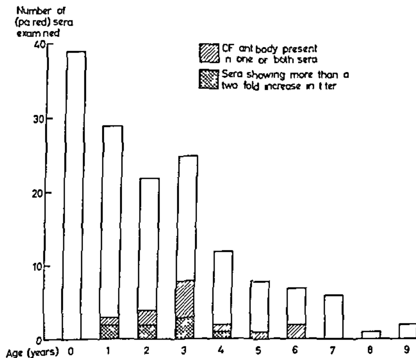


Fig 3

Distribution according to age of 151 paired sera from patients with acute respiratory disease in the Copenhagen area in the period September 1962 June 1963 All sera were examined for CF antibodies to the respiratory syncytial virus Those showing presence of antibody are indicated

TABLE 3

Complement Fixing Antibody Titres against the RS Virus, Age of Patients, Clinical Diagnosis and Month in which First Blood Sample is Obtained from 20 Infants and Children with Acute Respiratory Disease in Copenhagen during the Period September 1962 to June 1963

Patient no	CF titre		Age of patient	Clinical diagnosis	Month in which first blood sample is obtained
	I	II			
12920	< 4	4	3	Pneumonitis	September 1962
13115	16	< 4	3	„	October
13151	8	32	3		„
13360	32	8	3	Rhinopharyngitis	December
13483	<64	32	1	Pneumon infiltr	January 1963
13551	< 4	>64	2	Pneumonitis	„
13598	8	>64	4	„	„
13653	8	>64	3	„	February
13692	< 4	8	1	„	„
13711	< 4	32	1	„	„
13736	16	32	3	„	„
13815	>64	>64	2	„	March
13862	< 4	32	3	Pneumon infiltr	„
13869	< 4	8	2	Pneumonitis	„
14116	16	8	5	„	April
14124	32	16	6	Rhinopharyngitis	„
14146	16	16	3	Acute laryngitis	„
14196	8	8	2	Pneumonitis	„
14223	32	16	4	Acute bronchitis	„
14247	>64	32	6	Bronchopneumonitis	May

According to this data the RS virus seems to have played a part in the aetiology of the disease in question in 12 per cent of all the cases examined during January, February and March, 1963 From the other data in this part of our survey it is clearly seen that these 12 per cent represent an accumulation of cases, indicating that a wave of infections with this virus went through the community during the first three months of 1963

Incidence of CF Antibody to the Respiratory Syncytial Virus in Sera taken During Six Consecutive Years

In order to estimate whether the incidence of antibodies differed particularly during successive years, 268 single sera from the years 1957–1962 were examined They had all been found negative by the psittacosis complement fixation test, as is generally the case with the age-group concerned (0 9 years) Fig 4 shows the distribution of the 268 sera examined according to the years during which they were taken For each year the patients are divided into two-age-groups, 0–4 and 5–9 years The number of sera showing the presence of antibodies is also indicated When the total numbers of seropositives from two successive years are compared by the chi square test, no statistically significant

fections (Patient 13711 also had thrombocytopenic purpura and patient 13598 associated croup) None of the 20 patients had a CF antibody rise to influenza A, B or C, and only one (13115) had a CF antibody rise to adenovirus

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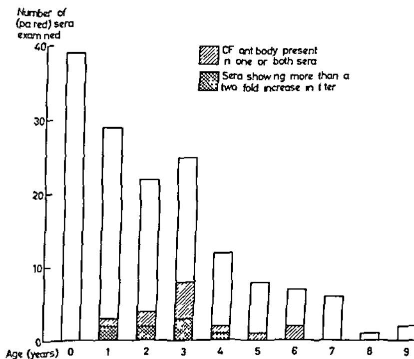


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13692	< 4	8	1	"	"
13711	< 4	32	1	"	"
13736	16	32	3	"	"
13815	>64	>64	2	"	March
13862	< 4	32	3	Pneumon infiltr	"
13869	< 4	8	2	Pneumonitis	"
14116	16	8	5	"	April
14124	32	16	6	Rhinopharyngitis	"
14146	16	16	3	Acute laryngitis	"
14196	8	8	2	Pneumonitis	"
14223	32	16	4	Acute bronchitis	"
14247	>64	32	6	Bronchopneumonitis	May

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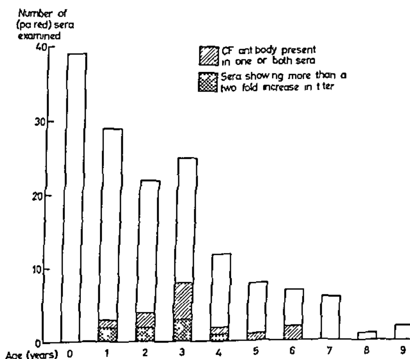


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13711	< 4	32	1		
13736	16	32	3		
13815	>64	>64	2		March
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(Head Arne Lithander, M D)

CONCENTRATION OF PENICILLIN IN HUMAN PLASMA

Investigation of the Relation to the Content of Albumin and of Total Protein in Plasma

By

ARNE LITHANDER

Received 27 ii 64

Chemotherapeutic preparations are known to be bound to blood plasma in varying degree The extent to which this occurs differs from one preparation to another It also differs for the plasma of different animal species In the case of penicillin, research has revealed a considerable degree of binding to plasma and wide variations between different kinds of penicillin *Klein* (1), for example, stated that the activity of penicillin in human plasma was only 50 per cent of that in buffer solution *Tompsett et al* (2) reported that penicillin K lost 85 to 90 per cent of its activity in 30 per cent human serum, while penicillin G lost 40 to 60 per cent of its activity when similarly tested *Smith et al* (3) found that the degree of binding of penicillin V by human plasma was significantly higher than that of penicillin G *Chow & McKee* (4) showed that penicillin was bound by human albumin in water solution at the concentration present in normal human plasma *Lithander* (5) reported that benzyl penicillin activity was relatively more reduced by rabbit plasma when the penicillin concentration was low than when it was high

The following investigation was conducted to determine in what way the binding of penicillin is dependent on the concentration in human plasma of, for example, albumin

MATERIALS AND METHODS

Samples of venous blood were taken from 30 patients and mixed with a small amount of heparin None of the patients had been given antibacterial preparations Just prior to the removal of these samples an electrophoretic investigation was made of the concentration of total protein albumin and the different globulin fractions in plasma Immediately after the samples were taken the plasma was separated from the corpuscles by centrifugation Benzyl penicillin[1] was dissolved in plasma or dilutions of plasma so as to secure different concentrations The penicillin con

The penicillin in the present investigation was kindly supplied by AB Kabi Stockholm Sweden

1 Hereinafter the term benzyl penicillin will be abbreviated to penicillin

centrations were 0.5, 0 25, 0 125 and 0 0625 IU per ml The plasma was used both undiluted and diluted with phosphate buffer solutions 1/2 5, 1/5, 1/10 and 1/20

Diffusion technique in agar was used to determine the penicillin content of the abovementioned solutions of penicillin Subtilis spores of the ATCC 6633 strain were used as test organisms The spores were suspended in melted agar in a concentration of 7 500 spores per ml of agar The agar was poured into Petri dishes When it had solidified, seven cylindrical holes were punched out on each plate Then 0 06 ml of phosphate buffer solutions of standard penicillin containing 0 12, 0 06 and 0 03 IU per ml, respectively were introduced into three of the holes in each plate The remaining holes were filled with 0 06 ml of the penicillin solutions in plasma or dilutions thereof to be tested Five plates were used in each test Readings were made following incubation at 37° C for 18 hours Two diameters at right angles to the

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RESULTS

In Table 1 we find the concentrations of albumin and total protein in plasma Table
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Table 3 also contains the correlations between the concentration of unbound penicillin and the content of the total protein of the plasma

TABLE 1

Concentration of Albumin and Total Protein in Plasma
Means Are Expressed in gm/100 ml
Concentration in plasma

	n	$\bar{x} \pm \sigma_{\bar{x}}$	σx	v
Albumin	30	3 7 ± 0 22	1 22	33 0
Total protein	30	7 3 ± 0 20	1 09	15 0

The concentrations of albumin varied between 1 44 and 6 71 and those of total protein between 4 50 and 9 78 gm/100 ml

It appears from Table 2 and Fig 1 that there was a

demonstrated in dilutions of plasma also It was relatively greater the lower the concentration of penicillin administered When

TABLE 2

Yield of Unbound Penicillin in Different Dilutions of Human Plasma
Yield Is Expressed in per Cent of the Concentration of the Penicillin Dose

Concentration of penicillin dosage in plasma IU per ml	Dilution of plasma	n	Yield of unbound penicillin in plasma			
			$\bar{x} \pm \sigma\bar{x}$	σx	v	t
0 5	Undiluted	30	80 2 ± 1 54	8 42	10 5	−12 853
	1/2 5	30	87 8 ± 1 53	8 36	9 5	− 7 973
	1/5	30	92 0 ± 1 47	8 04	8 7	− 5 457
	1/10	30	94 8 ± 1 33	7 30	7 7	− 3 879
	1/20	30	98 3 ± 1 08	5 89	6 0	− 1 544
0 25	Undiluted	30	74 3 ± 1 50	8 24	11 1	−17 095
	1/2 5	30	82 4 ± 1 38	7 54	9 2	−12 777
	1/5	30	86 9 ± 1 23	6 73	7 8	−10 693
	1/10	30	89 9 + 1 04	5 71	6 4	− 9 688
	1/20	30	93 1 ± 0 81	4 43	4 8	− 8 531
0 125	*Undiluted*	*30*	*71 6 ± 1 30*	*7 10*	*10 0*	*−21 878*
	1/2 5	30	80 5 ± 1 03	5 66	7 0	−18 889
	1/5	30	86 8 ± 0 92	5 06	5 8	−14 244
	1/10	30	90 1 ± 0 90	4 93	5 5	10 965
	1/20	30	91 6 ± 0 72	3 93	4 3	11 721
0 0625	Undiluted	30	61 1 ± 5 88	32 2	52 7	− 6 623
	1/2 5	30	75 9 ± 1 47	8 03	10 6	−16 459
	1/5	30	84 1 ± 1 16	6 33	7 5	−13 775
	1/10	30	88 9 ± 0 81	4 45	5 0	−13 649
	1/20	30	92 9 ± 0 63	3 45	3 7	11 272

the concentration of penicillin was 0 5 IU per ml, the plasma had no effect in a dilution of 1/20 With lower concentrations of penicillin, plasma in a dilution of 1/20 did have a significant effect

Table 3 reveals that the binding of penicillin was significant, depending on the concentration of albumin and protein in plasma The correlation to the concentration of albumin was more pronounced than to the concentration of total protein The yield in per cent of unbound penicillin decreased with increasing albumin content The penicillin yield in undiluted plasma decreased by an average of 4 per cent with an increase in albumin content of 1 per cent Fig 2 illustrates that the yield of penicillin with different calculated penicillin concentrations depended on the concentration of albumin in undiluted plasma The slope of the regression lines is about the same for the four penicillin dosages used

Comparisons between the results of penicillin determinations in plasma or dilutions thereof and the concentration of the various globulin fractions in plasma revealed no significant relation between the concentration of unbound penicillin, on the one hand, and the concentration of any globulin fraction on the other

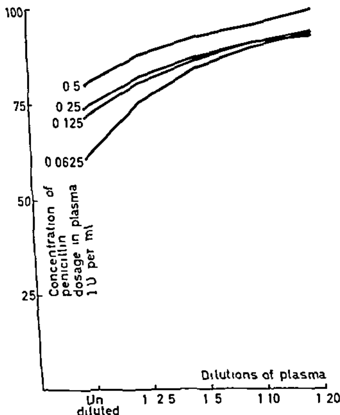


Fig 1

Yield of unbound penicillin in different dilutions of human plasma Yield is expressed in per cent of the concentration of the penicillin administered The results (means) are the same as in Table 2

COMMENTS

In the treatment of infections with penicillin it is sometimes helpful to determine in advance the concentration of free penicillin which will be found in the patient s blood plasma The dosage of penicillin is adapted to suit the nature of the infection and in localized infections, the site of the infection Consequently it may be of interest to know whether the binding to plasma alters when the dosage of penicillin is varied

Several researchers have shown that penicillin is bound to plasma and also that albumin in concentrations corresponding to the concentration in normal human plasma binds penicillin

The present investigations revealed that the concentration of albumin

TABLE 2

Yield of Unbound Penicillin in Different Dilutions of Human Plasma
Yield Is Expressed in per Cent of the Concentration of the Penicillin Dose

Concentration of penicillin dosage in plasma IU per ml	Dilution of plasma	n	Yield of unbound penicillin in plasma			
			$\bar{x} \pm \sigma_{\bar{x}}$	σx	v	t
0.5	Undiluted	30	80.2 ± 1.54	8.42	10.5	−12.853
	1/2.5	30	87.8 ± 1.53	8.36	9.5	− 7.973
	1/5	30	92.0 ± 1.47	8.04	8.7	− 5.457
	1/10	30	94.8 ± 1.33	7.30	7.7	− 3.879
	1/20	30	98.3 ± 1.08	5.89	6.0	− 1.544
0.25	Undiluted	30	74.3 ± 1.50	8.24	11.1	−17.095
	1/2.5	30	82.4 ± 1.38	7.54	9.2	−12.777
	1/5	30	86.9 ± 1.23	6.73	7.8	−10.693
	1/10	30	89.9 ± 1.04	5.71	6.4	− 9.688
	1/20	30	93.1 ± 0.81	4.43	4.8	− 8.531
0.125	Undiluted	30	71.6 ± 1.30	7.10	10.0	−21.878
	1/2.5	30	80.5 ± 1.03	5.66	7.0	−18.889
	1/5	30	86.8 ± 0.92	5.06	5.8	−14.244
	1/10	30	90.1 ± 0.90	4.93	5.5	−10.965
	1/20	30	91.6 ± 0.72	3.93	4.3	11.721
0.0625	Undiluted	30	61.1 ± 5.88	32.2	52.7	− 6.623
	1/2.5	30	75.9 ± 1.47	8.03	10.6	−16.459
	1/5	30	84.1 ± 1.16	6.33	7.5	−13.775
	1/10	30	88.9 + 0.81	4.45	5.0	13.649
	1/20	30	92.9 ± 0.63	3.45	3.7	11.272

the concentration of penicillin was 0.5 IU per ml, the plasma had no effect in a dilution of 1/20. With lower concentrations of penicillin, plasma in a dilution of 1/20 did have a significant effect.

Table 3 reveals that the binding of penicillin was significant, depending on the concentration of albumin and protein in plasma. The correlation to the concentration of albumin was more pronounced than to the concentration of total protein. The yield in per cent of unbound penicillin decreased with increasing albumin content. The penicillin yield in undiluted plasma decreased by an average of 4 per cent with an increase in albumin content of 1 per cent. Fig. 2 illustrates that the yield of penicillin with different calculated penicillin concentrations depended on the concentration of albumin in undiluted plasma. The slope of the regression lines is about the same for the four penicillin dosages used.

Comparisons between the results of penicillin determinations in plasma or dilutions thereof and the concentration of the various globulin fractions in plasma revealed no significant relation between the concentration of unbound penicillin, on the one hand, and the concentration of any globulin fraction on the other.

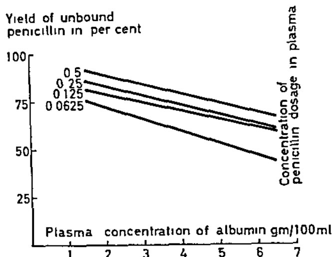


Fig 2

Relations between the concentration of unbound penicillin and the content of albumin in undiluted plasma

and total protein played an important part in the binding of penicillin when the dosage of penicillin varied between 0 06 and 0 5 I U per ml of plasma or dilutions thereof The degree of binding varied with the penicillin level in plasma The capacity to bind penicillin was diminished when the plasma was diluted but it was appreciable even in relatively highly diluted plasma when the level of penicillin was low

SUMMARY

1 The binding of benzyl penicillin to human plasma was analysed with respect to the importance of the concentration of albumin and total protein

2 The binding of benzyl penicillin was related to the concentration of albumin and total protein The correlation to albumin was more pronounced than to total protein This binding capacity of human plasma could be demonstrated up to a plasma dilution of 1/20

3 In undiluted plasma the concentrations of unbound penicillin decreased on an average of 4 per cent for each 1 per cent increase in the albumin content

REFERENCES

1 *Kle n I* Bnl

2 *Tomp*

TABLE 3

Correlations between the Concentration of Unbound Penicillin and the Concentration of Plasma Albumin and of Total Protein with Different Doses of Penicillin and Different Dilutions of the Plasma

Concentration of penicillin dosage in plasma IU per ml	Dilutions of plasma	Correlations			
		Albumin		Total protein	
		b ± εb	r ± εr	b ± εb	r ± εr
0.5	Undiluted	4.92 ± 0.910	0.71 ± 0.089	−2.88 ± 1.350	−0.37 ± 0.157
	1/2.5	−4.13 ± 1.030	−0.61 ± 0.115	−2.21 ± 1.390	−0.29 ± 0.167
	1/5	3.74 ± 1.015	−0.57 ± 0.123	−2.36 ± 1.310	−0.32 ± 0.164
	1/10	3.40 ± 0.926	−0.57 ± 0.123	−2.07 ± 1.200	−0.31 ± 0.165
	1/20	2.27 ± 0.802	−0.47 ± 0.142	−2.56 ± 0.898	−0.47 ± 0.142
0.25	Undiluted	−5.14 ± 0.823	−0.76 ± 0.076	−4.15 ± 1.190	−0.55 ± 0.126
	1/2.5	3.92 ± 0.899	−0.64 ± 0.109	−3.61 ± 1.120	−0.52 ± 0.133
	1/5	3.07 ± 0.862	−0.56 ± 0.126	−3.07 ± 1.001	−0.50 ± 0.137
	1/10	−2.53 ± 0.741	−0.54 ± 0.129	−2.84 ± 0.830	−0.54 ± 0.129
	1/20	−1.24 ± 0.642	−0.34 ± 0.161	−1.85 ± 0.682	−0.46 ± 0.145
0.125	Undiluted	4.74 ± 0.631	−0.82 ± 0.061	−3.28 ± 1.060	−0.50 ± 0.136
	1/2.5	2.59 ± 0.723	−0.61 ± 0.115	−2.69 ± 0.837	−0.52 ± 0.132
	1/5	2.03 ± 0.680	−0.49 ± 0.138	−2.44 ± 0.745	−0.53 ± 0.132
	1/10	1.74 ± 0.686	−0.43 ± 0.148	−1.84 ± 0.779	−0.41 ± 0.152
	1/20	1.55 ± 0.531	−0.49 ± 0.140	−1.08 ± 0.649	−0.30 ± 0.166
0.0625	Undiluted	6.53 ± 0.973	−0.79 ± 0.070	−4.77 ± 1.510	−0.51 ± 0.135
	1/2.5	−4.56 ± 0.904	−0.69 ± 0.096	−3.58 ± 1.230	−0.48 ± 0.140
	1/5	−2.83 ± 0.817	−0.57 ± 0.128	−2.72 ± 0.986	−0.47 ± 0.142
	1/10	−1.54 ± 0.622	−0.42 ± 0.150	−0.94 ± 0.749	−0.23 ± 0.173
	1/20	−1.07 ± 0.493	−0.38 ± 0.157	1.04 ± 0.564	−0.33 ± 0.163

b = regression coefficient
r = correlation coefficient

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The International Escherichia Centre Statens Seruminstitut Copenhagen Denmark
Central Veterinary Laboratory Weybridge England and
Veterinaruntersuchungs und Tiergesundheitsamt Dresden Germany

K ANTIGENS K88ab(L) AND K88ac(L) IN *E coli*

A New O Antigen O147 and a New K Antigen K89(B)

By

IDA ØRSKOV FRITS ØRSKOV W J SOJKA and W WITTIG

Received 8 v 64

In a previous paper (10) the existence of *E coli* strains with two kinds of K antigens a B antigen and an L antigen was reported A description was given of two strains G7 and F68 isolated from swine which possessed different O B and H antigens but the same L antigen named K88 It was shown that the detection of this L antigen was simplified by the fact that it was not developed after growth at 18° C

At first the K88 antigen was thought of as an entity as the two strains mentioned above had identical L antigens Since then additional types with the K88 antigen were encountered among strains isolated from piglets in different countries Investigation of such strains seemed to indicate that the K88 antigen was composed of several antigenic factors which could be present in different combinations The present report deals with the result of these investigations

MATERIAL AND METHODS

Strains

The m

[illegible] ur strains above the line were isolated during the years 1960–63 G1253 G205 and G491 originated from England and were first analysed by *Sojka* while strain CS1599 came from Germany and was first examined by *Wittig* in Dresden A strain *(C662 63)* of the *O138 K81* type with the K88(L) antigen was received from Dr *Wittig* before G491 but was not investigated further

Preparation of Antisera

These were prepared by injecting rabbits five times at intervals of two or four to five days In the first case the doses ranged from 0 1 to 1 0 ml in the second from 0 2 to 2 0 ml The animals were bled seven or eight days after the la[illegible]

1

details of technique see (10)

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4 *Chow B F & McKee C M* Interaction between crystalline penicillin and human plasma proteins Science *101* 67 1945
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TABLE 2

Results of Slide Agglutination Test

Antigens	Growth temp °C	Sera: OK E68 — test K88 (L): Unabs	Sera: OK E68 — test K88 (L): Abs by live G1253	Sera: OK G1253: Unabs	Sera: OK G1253: Abs by live G7
G7	37	+++	+++	+++	—
G7	18	—	—	—	—
G1253	37	+++	—	+++	+++
G1253	18	—	—	+++	+++

The recording of a positive reaction as +++ means that the clumping is heavy

TABLE 3

Results of Tube Agglutination

Antigen	Cultiv temp °C	Sera: Test K88 (L)	Sera: OK G1253: Unabs	Sera: OK G1253: Abs by live G7	Sera: OK G1253: Abs by homol 100 C cult = pure L serum	Sera: O G1253
G7	37	1280	320	0	320	0
G7	18	0	0	–	–	–
G1253	37*	160	640	160	320	80
G1253	18	0	320	160	0	40
G205	37*	320	320	640	320	0
G205	18	0	0	–	0	–
G491	37	160	320	320	320	0
G491	18	0	0	–	0	–

* These cultures agglutinated to the same extent in the saline control tube, revealed by means of hand lens only

Titre is the reciprocal value of the highest serum dilution showing clumping visible to the naked eye

strable if the culture has been grown at 18° C This fact is indicative of the presence of K88 antibodies in OK serum G1253 Similarly, only the 37° C culture of G1253 reacts in serum K88, which points to the same fact, that is that the L antigen K88 is present in this strain Since G1253 is incapable of removing the antibodies from K88 serum reacting with culture G7, the K88 antigen of the two strains cannot be identical From the table it is not evident whether G7 can absorb the K88 antibodies from the OK serum G1253, as culture G1253 agglutinates in the absorbed serum regardless of cultivation temperature As this reaction might be a B (or an O) antigen reaction, the serum has to be depleted of B and O antibodies by saturation with the homologous boiled culture before absorption with live culture of G7 Before showing the results of such absorptions in Table 4, we show the results of some tube agglutination tests in Table 3, strains G205 and G491 are

TABLE 1
Strains Used

Strain no	Antigens O	K B	K I	H	Haemolysis	Received from
G7	8	87	*88ab*	19	+	Sojka Weybridge England
E68	141	85ab	*88ab*	4	+	„ „
G1253	147	89	*88ac*	19	+	„ „
G205	8	87	*88ac*	19	+	„ „
G491	138	81	88ac	19	—	
CS1522	(1 117)	n e	88ac	10	—	Wittig Dresden Germany
G1108F	141	85ac	88ab	4	+	Sojka Weybridge, England
C258 61	147	n e	88ac	19	+	Thal Stockholm, Sweden
C794 61	147	n e	88ac	19	+	Sorum Oslo, Norway
C789 61	*8*	*87*	*88ac*	*19*	+	„
C105 62	147	n e	88ac	19	+	Wittig Dresden Germany
C462-62	147	n e	88ac	19	+	
C467-62	8	87	88ac	*?	+	„
C308 63	unknown	n e	88a?	43	—	
C662 63	138	81	88ac	19	+	
C667-63	(115)	n e	88a?	10	—	
C1160 63	*8*	*87*	*88ac*	*19*	+	*Djurisic, Novi Sad Jugoslavia*

*? means not motile enough for H determination
n e = not examined
Only K88 factors in italic have been fully verified experimentally

RESULTS

The K Antigen of the L Type

The three new English strains included in this study, all of different O antigen type, had one particular property in common they very often agglutinated in saline when tested as live culture grown at 37° C, but the agglutination varied in extent from time to time After heating to 100° C for one hour the autoagglutinability disappeared, and it was *never present in live cultures grown at 18° C Because of the instability* in saline the results of the agglutination were difficult to interpret in some cases, but in others it was thought justifiable to rely upon *an agglutination in serum if the intensity of this was unquestionably* greater than that of the control Slide agglutination was generally more useful than tube agglutination

O and OK sera produced from strain G1253 were used throughout the study In addition OK sera prepared with the previously described G7 and E68 strains were employed As will be remembered, these two strains have identical K88(L) antigens As one of the cultures studied, G205, had O, B and H antigens similar to G7, only the reactions in the E68 OK serum are presented here

In Table 2 it is shown that G7 agglutinates in the OK serum G1253 in the same manner as in the OK serum E68, designated here and in the following tables as test K88(L), i e no agglutination is demon-

TABLF 2

Results of Slide Agglutination Test

Antigens	Growth temp °C	Sera			
		Ok I 68 — test K88 (L)		OK G1253	
		Unabs	Abs by live G1253	Unabs	Abs by live G7
G7	37	+++	+++	+++	—
G7	18	—	—	—	—
G1253	37	+++	—	+++	+++
G1253	18	—	—	+++	+++

The recording of a positive reaction as +++ means that the clumping is heavy

TABLE 3

Results of Tube Agglutination

Antigen	Cultiv temp °C	Sera				
		Test K 88 (L)	OK G1253			O G1253
			Unabs	Abs by live G7	Abs by homol 100 C cult = pure L serum	
G7	37	1280	320	0	320	0
G7	18	0	0	–	–	
G1253	37*	160	640	160	320	80
G1253	18	0	320	160	0	40
G205	37*	320	320	640	320	0
G205	18	0	0	–	0	–
G491	37	160	320	320	320	0
G491	18	0	0	–	0	

* These cultures agglutinated to the same extent in the saline control tube revealed by means of hand lens only

Titre is the reciprocal value of the highest serum dilution showing clumping visible to the naked eye

strable if the culture has been grown at 18° C This fact is indicative of the presence of K88 antibodies in OK serum G1253 Similarly, only the 37° C culture of G1253 reacts in serum K88, which points to the same fact that is that the L antigen K88 is *present in* this strain Since G1253 is incapable of removing the antibodies from K88 serum reacting with culture G7, the K88 antigen *of the* two strains cannot be identical From the table it is not evident whether G7 can absorb the K88 antibodies from the OK serum G1253, as culture G1253 agglutinates in the absorbed serum regardless of cultivation temperature As this reaction might be a B (or an O) antigen reaction, the serum has to be depleted of B and O antibodies by saturation with the homologous boiled culture before absorption with live culture of G7 Before showing the results of such absorptions *in Table 4*, we show the results of some tube agglutination tests in Table 3, strains G205 and G491 are

included. *From this it appears that strain G1253* has an L antigen, as was to be anticipated from Table 2, since absorption by homologous boiled culture leaves antibodies reacting with the live 37° C culture The 18° C culture is negative in this pure L serum, just as it is negative in the test K88 serum. In addition, the table shows that strains *G205 and G491 also have a K88* antigen complex, since 37° C cultures agglutinate both in the pure L serum of G1253 and in the test K88 serum. Since they also agglutinate in OK serum G1253 after absorption of this with G7, their K88 antigen is not identical with that of G7

TABLE 4

Results of Slide Agglutination Tests

Antigens	Sera							
	Test K88(L)				Pure L of G1253			
	Unabs	Abs by live cult of			No further abs	Further abs by live cult of		
		G1253	G205	CS1522		G7	G205	CS1522
G7	+++	+++	+++	+++	+++	—	—	+
G1253	+++	—	—	+	+++	+++	—	+
G205*	+++	—	—	+	+++	+++	—	+
G491†	+++	?	?	?	+++	+++	?	?
CS1522	+++	—	—	—	+++	+++	—	—

All cultures were grown at 37° C
18° C cultures of all strains including CS1522 reacted as shown in the preceding tables, and are therefore omitted

* This culture showed clumping in saline which was visible only with the aid of a hand lens
† The clumping in saline of this culture was visible to the naked eye
+++ versus + means difference in degree of agglutination

Additional absorptions of OK serum G1253 absorbed by homologous 100° C culture, *i e* the pure L serum, were thereafter carried out In three separate experiments live cultures of G7, G205 and CS1522 were *used as absorbing agents Strain CS1522* was *included in the study* as it was not autoagglutinable and probably had a K88 antigen resembling that of G1253 The test K88 serum was absorbed by the same cultures The results of slide agglutination tests performed with these absorbed sera are shown in Table 4 From this it is evident that the K88(L) antigens of G7 and G1253 have an ab, ac relationship This is deduced from the information, also presented in Table 2 regarding the absorption of the test K88 serum by culture G1253, and from the fact, as shown in Table 4, *that G7 is incapable of removing the L antibodies* from the pure L serum of G1253 The K88 antigen factors of the strains in question can then be expressed in the following way

K88ab(L)	F68
K88ab(L)	G7
K88ac(L)	G1253
K88ac?(L)	G205
K88(a)c?(L)	CS1522

As no sera of strains G205 and CS1522 were examined in this study, nothing is known about additional K88 factors of these strains This is expressed by a question-mark (?) in the formulas Strain CS1522 seems to have a K88 antigen which closely resembles that of G1253, but the absorptions indicate that the a factor is not complete. For this reason it is placed in brackets No formula is given for G491, as this culture was not used as absorbing culture, and in the experiments presented in Table 4 it was autoagglutinable In Table 1 the K88 antigen of G491 is given as K88ac

Following the finding of the K88ac(L) antigen factors in both strain G1253 (0147) and strain G205 (08), 53 strains considered to be, and labelled as, "G7" types were re-examined in Weybridge by one of the authors By slide agglutination technique using OK serum of G1253 absorbed with the G7 strain, it was shown that only 43 per cent of the strains were of the "G7" type, *i e* they had the K88ab factors, while the remaining strains were of the "G205" type In Table 1 a list is given of strains with K88(L) antigen received in the International Escherichia Centre Each strain is a representative of several strains from the same laboratory

In the G7 strain itself some sort of an antigenic variation from the b to the c factor of K88 has been looked for, at one time 670 single colonies originating from one colony were checked in absorbed sera, but none was found to have the ac factors

As stated previously (9), strain G7 is a genetic donor, *i e* when G7 is seeded with another coli strain the latter acquires the K88(L) antigen and the recipient strain can furthermore now act as donor in genetic crosses Up to the present it has not been possible to demonstrate the transfer of the K88ac(L) antigen from either G1253 or G205 to a recipient (H509a), which in a parallel experiment was capable of receiving the K88ab(L) antigen from G7

Other Antigens

For information of the other antigens possessed by the strains, the reader is referred to the sero formulas given in Table 1 The determination of these antigens was carried out by standard procedures When the O, B and H antigens are assigned to known types this does not mean that the identity with antigens of test types has been demonstrated by serum production and cross absorption, but that agglutination tests have showed reactions which justified the serotypes given

One new O antigen (0147) was established This antigen has some relationship to the O groups already established, particularly to 0133,

013, 0139 and 0102 At first it was not considered desirable to let strain G1253 represent a new O group, as it might be regarded as belonging to O group 133 However, many strains of this group, isolated from diseased swine in different countries, were received in the International Escherichia Centre, and it thus seemed to be a common swine strain In some cases the strains agglutinated only at low titre in the anti 0133 serum This means that the reaction might be overlooked Moreover, it was shown in reciprocal cross absorption tests that strain G1253 and test strain for O antigen 133 had special O antigen factors For these reasons it was decided to establish 0147 as a new O antigen

As the L antigen of culture G1253 is suppressed by 18° C cultivation, inagglutinability in O serum of an 18° C culture is presumably caused by the presence of a K antigen of the B type The 18° C culture agglutinated well in the OK serum and could, after boiling, remove all agglutinins reacting with the 18° C culture (see Table 5) This means that the strain has a B antigen This B antigen was numbered K89(B), as it was not found to be related to any of the K antigens established previously, except that the test strain of K21 agglutinated to some extent in the OK serum G1253 The reverse test was negative

TABLE 5
Demonstration of a B Antigen at G1253

Antigens	Sera		
	O G1253	OK G1253	
		Unabs	Abs by boiled G1253 18°C
Formalinized G1253 18° C	20	160	0
Heated to 100° C G1253 18° C	*1280*	*1280*	*0*

The O antigen of strain CS1522 has been designated (01,0117), which means that it has some relationship to both these O groups A number of strains of this type have been received during the last two or three months, all from one laboratory (Dr Wittig, Dresden) Future findings will show whether also this type deserves its own O antigen number Strain CS1522 was not examined for B antigen

The H antigens especially represented among the strains are H4, H19 and H10 It is noteworthy that the 08, the 0138 and the 0147 strains all had H antigen 19 Common to strains with the K88(L) antigen and the H19 antigen is the fact that they are poorly motile For this reason the H determination can be time-consuming and at first apparently unfeasible H10 was found in two strains of different O groups (see Table 1)

At this juncture it would be appropriate to mention the peculiar fact that the patterns of the fermenation reactions seem to follow the H

antigens (Table 6) Three different patterns were found corresponding to the three different H antigens Even those strains mentioned above, which had H10 but different O antigens, had the same fermentation pattern It is also to be noted that these H10 strains are able to decompose urea, and that 0141 K85 H4 strains are also often urea positive It is evident from the table that most of the strains are haemolytic

TABLE 6

Biochemical Reactions of the Strains Examined

	E68 0141 K85 H4	G7 08 K87 H19	G205 08 K87 H19	G1253 0147 K83 H19	G491 0138 K81 H19	CS1522 (01 0117) H10
Adonitol	—	—	—	—	—	+
Dulcitol	+	+	+	+	+	—
Sorbitol arabinose xylose rhamnose maltose	+	+	+	+	+	+
Salicin	+[3]	—	—†	—†	+[6]	+[7]
Inositol	—	—	—	—	—	—
Lactose	+	+	+	+	+	+
Sucrose	—	+	+	+	+	—
Mannitol glucose	++	++	++	++	++	++
Sorbose	—	+	+	+	+	+
Urea	—*	—	—	—	—	+
Haemolysis°	+	+	+	+	—‖	—§

As all strains had the K88(L) antigen this is omitted from the seroformulas given
* Some strains of this type are urea positive
† Both salicin negative and late positive strains of these serotypes have been found
§ One of 21 strains received almost simultaneously was haemolytic
° Haemolysis tested on 5 per cent sheep blood agar plates
‖ 0138 strains from Germany were haemolytic
\+ means acid ++ menas acid and gas

DISCUSSION

The first strains with the L antigen K88 were isolated from swine in England Not only was this L antigen detected in a strain which already had a K antigen of the B type but the same L antigen was also shown in another strain which differed from the first in respect of O B and H antigens

It was later demonstrated that strains having O, B and H antigens (08, K87 H19) similar to one of the two above-mentioned strains, also had the K88 antigen, but of a somewhat different composition The first K88 antigen to be described was now called K88a, b, whilst the later one was symbolized K88a, c The fact that apparently identical strains sometimes have the ab and sometimes the ac factors is rather remarkable and leads one to suspect that an antigen variation might take place in the separate cultures, leading to the change from one determinant group of the antigen molecule to the other This variation phenomenon is well known from the Salmonella serology (4)

Variation in the B antigen (or OB complex) in *E coli* has been shown previously, but it has hitherto not been possible to demonstrate this type of variation in the K88 antigen, at least not in the G7 strain Additional experiments are being carried out

Another fact worth noticing is that the K88ac factors have been found in several strains of still other serotypes from swine, one of which (0138) is known to be connected with diseases in swine (1) It is known that certain diseases are characterized by certain *E coli* serotypes, *e g* some types are more frequently isolated from the intestinal tract of infants with diarrhoea than from other sources, some are especially isolated from swine with oedema disease (7), some from calves with white scours (8), and the types normally found in the intestinal tract also seem to differ somewhat from animal to animal etc However, the fact that the L antigen can be associated with a variety of serotypes found in swine is peculiar In some cases the strains were sent to the International Escherichia Centre because they were primarily shown to agglutinate in a K88 serum, in other words they were selected because of the K88 antigen, whilst in other cases the strains had not been primarily identified serologically at the place of isolation No investigation has been carried out to determine whether strains with the L antigen K88 can be isolated from sources other than the pig, but it appears from the present study that this L antigen seems to be quite widespread where swine are present, and to be something which can be fixed in different bacterial hosts This is perhaps not surprising, as it was already known that G7, at least, when mixed with another strain (H509a, not of swine origin), transmits the L antigen to this strain This ability was not demonstrated in strains with the K88ac factors in the cases examined Further experiments on these lines will be published later

The clumping in saline of live cultures of some strains with the L antigen K88 is reminiscent of a similar autoagglutinability in other strains with intact O antigen Some workers have claimed that K12 and derivatives of this strain clump when they are in the F^+ state (6), from the point of view of an O antigen analysis they are also claimed to be rough, *i e* they lack the O antigen and are autoagglutinable in saline after boiling (9) When the R factor R_{100-1} is introduced into a derivative of *E coli* H509a this becomes autoagglutinable, but this property disappears after boiling (2) In these cases the instable live cultures have been shown to harbour an episome (3) The determinant for the K88(L) antigen is probably carried by an episome or a plasmid (5) (unpublished results)

SUMMARY

The present study concerns serological examination of strains isolated from swine and shows that

1) The previously described L antigen K88 exists in at least two varieties one of which is symbolized K88ab the other K88ac
2) After cultivation at 18° C the K88ac antigen cannot be demonstrated (the same was previously shown to be true of the K88ab antigen)
3) The K88ac antigen has been found in several serological types which differed especially as to O and B antigens
4) Strains with the K88ac(L) antigen are geographically widely distributed
5) In preliminary experiments it was impossible to show the transferability of the K88ac antigen (the K88ab factor can be transferred to other strains)
6) The O and B antigens of one of the strains studied were established as new types and numbered O147 and K89(B)

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10 *O*

Acta path et microbiol scandinav 62, 448–458 1964

Statens Seruminstitut, Copenhagen and The Danish Atomic Energy Commission
Research Establishment, Risø Denmark

RADIATION RESISTANCE OF *STREPTOCOCCUS FAECIUM* AND SPORES OF *BACILLUS SUBTILIS* DRIED IN VARIOUS MEDIA

By

EBBE AHRENSBURG CHRISTENSEN and KNUD SEHESTED

Received 9 vii 64

Sterilization of disposable items of hospital equipment by means of ionizing radiation has become increasingly employed during the last few years (15, 16)

The calculation of the dose necessary for radiation sterilization of a given group of materials must take into account the greatest possible resistance of the micro-organisms under the given conditions It is a well-known fact, however, that the resistance of various micro-organisms to ionizing radiation may be altered by variations in the environment before, during, and after the irradiation (8) It must therefore be kept in mind that much of the available information about the resistance of micro-organisms to ionizing radiation is based on the resistance of organisms that have undergone procedures which, *a priori*, cannot be regarded as insignificant for their radiation resistance

The present study is part of a series of investigations of the resistance of various dried micro-organisms to ionizing radiation The resistance of two strains of *Streptococcus faecium* and of spores of two strains of *Bacillus subtilis* was investigated before and after the micro organisms had been subjected to some of the procedures commonly used in bacteriological technique

MATERIAL AND METHODS

The strains of *Str faecium* employed were the two which in a previous study on enterococcal strains (5) were found to be most radiation resistant One was a laboratory strain (O_{12}) and the other was a sub strain of a bacterium found in dust from a room housing an electron accelerator used for industrial radiation sterilization (F_t)

The strains were cultured on blood agar at 37° C for two days The cultures were then scraped off the plates and suspended in serum broth and isotonic phosphate buffered saline (pH 7 38) respectively Test pieces for the radiation experiments were made immediately after suspension by drying the suspension as drops (0 01 ml) on polyethylene foil at room temperature in atmospheric air (6)

As previously mentioned two spore forming strains of *B subtilis* were used One was the Serum Institute test strain for control of autoclaving and dry heat sterilization This strain will be designated as *B subtilis* in the present study The other

spore forming strain was *B globigii* ATCC 9372 (B subtilis var niger), which will be designated *B globigii* Dried, crude, spore preparations were made as described in a previous study (6) One of the preparations of B subtilis spores however, was made from a culture grown at 45° C for five days instead of the usual two days at 37° C and a further three days at room temperature

Cleaning of B subtilis spores was performed by suspending the unwashed dry, spore-containing powder in phosphate buffered saline and centrifuging the suspension at 3000 r p m for 30 minutes This procedure was performed three times, whereupon the sediment was resuspended in phosphate buffered saline, serum broth

a total of ten times The sediment was then dried at room temperature in atmospheric air and kept in air tight ampoules until use These washed globigii spores were resuspended in distilled water phosphate buffered saline and serum broth respectively Test pieces were made from the suspensions immediately after preparation and these test pieces were in every case dry 30 minutes at the most after the

were kept at + 4° C for 24 hours before use

In all cases the spore test pieces were made by drying the suspensions in drops (0 02 ml) on polyethylene foil at room temperature in atmospheric air

The heat resistance of the spores was used as criterion that the spores on the test pieces were actually spores and had not germinated during the preparation procedures In connection with the determinations of the number of viable units on the

used in the present investigation showed the same or a slightly higher, number of viable units than the test pieces which were not heated

Before and after irradiation the number of viable units per test piece was determined by suspension in phosphate buffered saline pH 7.38 or chilled serum broth

technique All counts of viable units

the Research Establishment of the

the electron accelerator facility or in

plant The dose in the electron accelerator facility was given with an accuracy of ± 5 per cent and in the Co^{60} plant with an accuracy of ± 2 per cent

RESULTS

In the *first experimental series*, two strains of *Str faecium* were examined for their resistance to ionizing radiation, after suspension and drying *in* serum broth, as well as after suspension and drying in phosphate buffered saline The test pieces compared were prepared from cultures *on* uniform plates, grown under uniform conditions, and the test pieces were prepared by a uniform technique

The resistance to ionizing radiation of both strains was less after suspension and *drying in buffered* saline than after suspension and drying in serum broth, and the reduction in resistance was of the same order of magnitude in both strains *(Fig 1)*

Test pieces prepared by suspension and drying of the bacteria *in* serum broth were stored at room temperature in atmospheric air for

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MATERIAL AND METHODS

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The strains were cultured on blood agar at 37° C for two days The cultures were then scraped off the plates and suspended in serum broth and isotonic phosphate buffered saline (pH 7 38) respectively Test pieces for the radiation experiments were made immediately after suspension by drying the suspension as drops (0 01 ml) on polyethylene foil at room temperature in atmospheric air (6)

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fug ... suspension at 8000 r p m for 30 minutes This procedure was performed a total of ten times The sediment was then dried at room temperature in atmospheric air and kept in air tight ampoules until use These washed globigii spores were resuspended in distilled water phosphate buffered saline and serum broth respectively Test pieces were made from the suspensions immediately after preparation and these test pieces were in every case dry 30 minutes at the most after the the spores had been resuspended The suspensions were kept at + 4° C for 24 hours and another batch of test pieces was then made from the suspensions Test pieces of unwashed globigii spores were prepared from suspensions of the spores in distilled water phosphate buffered saline and serum broth respectively All these suspensions were kept at + 4° C for 24 hours before use

In all cases the spore test pieces were made by drying the ...

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Before and after irradiation the number of viable units per test piece was determined by suspension in phosphate buffered saline pH 7 38 or chilled serum broth using the usual dilution and counting technique All counts of viable units was performed on blood agar

The irradiations were carried out at Risø the Research Establishment of the Danish Atomic Energy Commission either in the electron accelerator facility or in the Co^{60} plant The dose in the electron accelerator facility was given with an accuracy of + 5 per cent and in the Co^{60} plant with an accuracy of ± 2 per cent

RESULTS

In the *first experimental series*, two strains of *Str faecium* were examined for their resistance to ionizing radiation, after suspension and drying in serum broth as well as after suspension and drying in phosphate buffered saline The test pieces compared were prepared from cultures on uniform plates grown under uniform conditions and the test pieces were prepared by a uniform technique

The resistance to ionizing radiation of both strains was less after suspension and drying in buffered saline than after suspension and drying in serum broth and the reduction in resistance was of the same order of magnitude in both strains (Fig 1)

Test pieces prepared by *suspension and drying of the bacteria* in serum broth were stored at room temperature in atmospheric air for

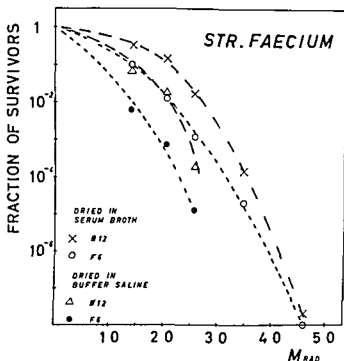


Fig 1
Inactivation curves of two strains of *Str faecium* (O_{12} and F_6) each strain was dried in serum broth and in buffered saline

50 days, and the resistance of the micro-organisms to ionizing radiation was then determined once more The radiation resistance was unaffected by storage, just as the number of viable units remained unchanged on the non-irradiated test pieces

Different enterococcal strains can show considerable differences in resistance to storage In some strains of *Streptococcus faecalis* the number of viable units per non irradiated test piece can fall almost 7 logarithmic steps in the course of 5 months while during the same period the greatest fall observed in the number of viable units on test pieces prepared with *Str faecium* was 3 logarithmic steps After storage for 5 months a number of the faecium strains studied in a previous investigation (5) showed an unchanged or almost unchanged number of viable units per test piece

In the *second experimental series*, washed and unwashed spores of *B subtilis* were examined for their resistance to ionizing radiation The spores were obtained from the same preparation, and the washed spores were treated as one lot until suspension and drying in the different media

Unwashed spores showed the greatest resistance to ionizing radiation after suspension and drying in serum broth (Fig 2) The resistance of the spores was reduced as a result of the washing in phosphate buffered

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in methanol had least resistance to ionizing radiation (Fig 3)

Storage of the washed spores as a suspension in serum broth at

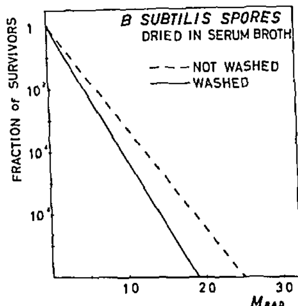


Fig 2
Inactivation curves of the same spore preparation of *B subtilis* before and after washing in buffered saline In both cases the spores were suspended in serum broth and dried in atmospheric air before irradiation

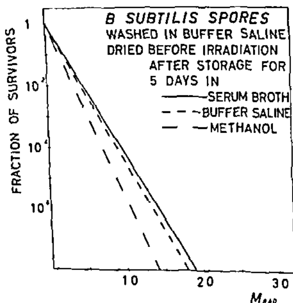


Fig 3

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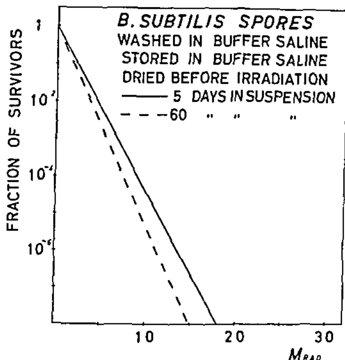


Fig 4
Inactivation curves of washed subtilis spores suspended in buffered saline at + 4° C for 5 days and 60 days respectively

+ 4° C for 60 days had no effect on the resistance of the spores to ionizing radiation Storage of the spores in buffered saline, on the other hand, might result in a reduction of the radiation resistance of the spores After storage for 5 days at + 4° C, spores stored and dried in serum broth showed the same resistance as spores stored and dried in buffered saline, but after storage for 60 days, the resistance of the spores suspended in buffered saline had declined (Fig 4)

Spores suspended and dried in methanol were, as mentioned, the least resistant ones in the experimental series (Fig 3), but their resistance was the same whether they had been suspended in the methanol for 5 days or 60 days This reduction in resistance was reversible If the spores were resuspended in serum broth after allowing the methanol to evaporate, they showed the same resistance to ionizing radiation as spores which had been suspended in serum broth for the entire period of storage (Fig 5)

Storage of the spores as suspensions at + 4° C in buffered saline, serum broth, or methanol caused no difference in the number of viable units per ml, whether the number of viable units was determined after heat activation at 80° C for 10 minutes, or whether the determination was made without this heat treatment

In the *third experimental series*, was examined whether culture at higher temperature and heat activation at 80° C for 10 minutes had any influence on the resistance to ionizing radiation of subtilis spores

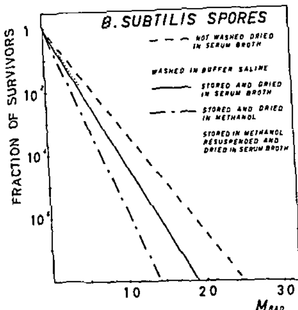


Fig 5

Inactivation curves of the same spore preparation of *B subtilis* The washed spores had been stored as suspensions at + 4° C for 60 days

Two spore preparations were made concurrently, the culture temperature being the sole difference in the experimental conditions One culture was grown at 45° C for 5 days, the other at 37° C for 2 days and at room temperature for 3 days Both radiation resistance and number of spores per mg of dry matter were the same in the two preparations The only difference which could be demonstrated was that in the preparation grown at 45° C, non-irradiated cultures germinated more rapidly and more uniformly on blood agar than in the preparation grown at the lower temperature This difference disappeared if the latter preparation was heat activated at 80° C for 10 minutes

In both preparations, heat activation before preparation of the test pieces was without influence on the resistance of the spores to irradiation Heat activation after irradiation was of no significance to the number of spores capable of multiplying, and seemed otherwise to be of no value, as both delay in colony formation and inequality of the colonies remained uninfluenced by heat activation at 80° C for 10 minutes in the experiments described here These two phenomena are always seen after irradiation with high doses, being the more pronounced the higher the dose Heat activation as well as plating were performed 24 hours at the earliest after irradiation, and thus, the heat-sensitivity of the spores was not examined immediately following irradiation

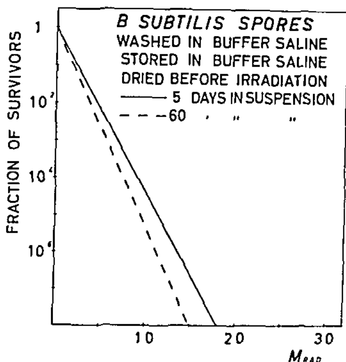


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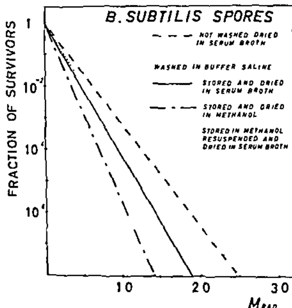


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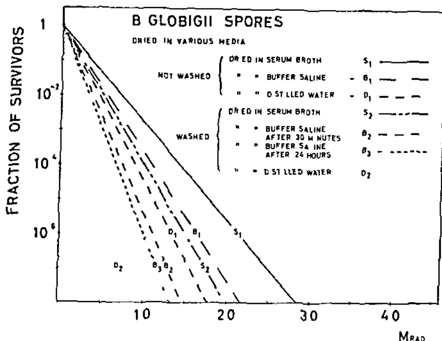


Fig 6

Inactivation curves of the same spore preparation of *B globigii* suspended and dried in various media

In the *fourth experimental series*, washed and unwashed spores of *B globigii* were examined for their resistance to ionizing radiation, after suspension and drying in various media The spores were from the same preparation, and were treated concurrently throughout the whole experimental series As mentioned, the cleaning of the globigii spores was carried out much more effectively than the cleaning of the subtilis spores used in the second experimental series

Just as in the second experimental series, cleaning of spores resulted in a reduction in their resistance to ionizing radiation (Fig 6) The only *outstanding difference between results from the second and the fourth* experimental series was that globigii spores suspended in buffered saline and dried within the first 30 minutes of suspension, showed a lower resistance to ionizing radiation than globigii spores suspended and dried in serum broth while, as mentioned, the less effectively washed subtilis spores had the same resistance after 5 days in the same two media

The washed globigii spores were therefore examined with a view to determining whether they had the same resistance after storage for 24 hours at + 4° C, and immediately after suspension This was found to be the case of globigii spores suspended and dried in serum broth, as well as of spores suspended and dried in distilled water, while the resistance of spores suspended and dried in buffered saline had decreased slightly (Fig 6)

DISCUSSION

The present study makes no attempt to contribute to an elucidation of the reasons why the radiation resistance of micro-organisms changes as a result of variations in outer conditions Some of these reasons are familiar Oxygen tension during and after irradiation is significant for the number of micro-organisms capable of multiplying (11, 20, 22) The water content of the organisms can also be of significance (17, 19, 20, 21), as well as the presence of various substances possessing the capacity of protecting the organisms (3)

The present studies were carried out, because in the literature on radiation sterilization (4, 10, 16), the values quoted for the resistance of various micro-organisms were low in comparison with the radiation resistance of the same organisms in the dry state, when determined by the technique used in the present study (6)

The probability of a number of micro-organisms surviving on material subjected to a sterilization procedure, is in the first instance determined by 1) the sort and number of micro-organisms on the material prior to the sterilization procedure, and by 2) the effect of the inactivating procedure at a point on the material at which the effect is at a minimum

When sterilization is carried out by autoclaving or by dry heat, the recommendation is to use temperature-time relations which ensure very great inactivation factors for the most heat resistant ones of the generally occurring, contaminating bacteria, even when these organisms are placed at points in the material where the effect of the sterilization procedure is at a minimum (13, 14) If the effect of radiation sterilization is to be equated with the effect of heat sterilization, the dose used for radiation sterilization must be given as a minimum value, as the difference between least dose and mean dose can be of considerable magnitude Furthermore, the effect of the irradiation must be calculated on the basis of the maximum resistance of the micro-organisms under the given conditions

As mentioned, the species of the micro-organisms which prior to sterilization may occur on the material is of considerable significance to the inactivation effect which can be achieved by a given sterilization procedure In radiation sterilization of dry plastic material, it may be permissible to disregard the possibility of an occurrence on the material of *Micrococcus radiodurans* (1), which has a very high radiation resistance in the dry state (6), as this micro organism so far has been demonstrated only in a few instances It has been found, however, that M radiodurans is not the only micrococcus which is more resistant than bacterial spores at doses applicable in radiation sterilization of plastics (7) As demonstrated in the present study, some strains of bacteria belonging to *Str faecium* have such a high radiation resistance if dried in serum broth, that the inactivation factor at 2 5 Mrad is only between $10^{?}$ and 10^{3} Str faecium is very widespread, and has a considerable

power of survival in the dry state If it is desirable to maintain the definition of "sterile" as *free from living micro-organisms*, it is necessary for the establishment of doses for radiation sterilization, to take into account the highly radiation-resistant vegetative bacteria

In England (16) and USA (2), 2 5 Mrad is used, both for sterilization of dry plastic material for use in hospitals, and for sterilization of suture material In defence of sterilization with 2 5 Mrad, it may be claimed that dry plastic material, as a result of the manufacturing procedure, may be considered contaminated only to a very slight degree before sterilization, always provided that the material is collected and packed under controlled, aseptic conditions, as contamination by dust or touch may involve the transfer of a considerable number of organisms (12)

If radiation sterilization is to give the same inactivation factor for Str faecium on dry plastic material as is usually recommended for test strains such as B subtilis or B *stearotermophilus* by autoclaving or for B globigii by ethylene oxide sterilization (12), then, based on present knowledge, *the minimum dose in radiation sterilization should be 4 5 Mrad* This dose is now being used for sterilization of disposable items of hospital equipment at Risø, the Research Establishment of the Danish Atomic Energy Commission

Were a presence of micro-organisms with a higher resistance to ionizing radiation to be observed at some future date on material to be sterilized by radiation, a standard of radiation sterilization which is in line with that for material autoclaved *lege artis*, could only be achieved 1) *if the dose can be further increased, or* 2) *if the initial contamination* can be controlled and kept at a low level, or 3) if the irradiation is combined with other methods of sterilization or disinfection

When sterilization is carried out by means of continous irradiation in a high power electron accelerator facility, the dose cannot be made much higher than 4 5 Mrad, as some of the types of plastic usually employed are intolerant to higher doses, when the dose is given in one operation In most cases, however, the damage to the material seems to be the result of the heat developed during irradiation with these high dose-rates Intermittent irradiation allows the temperature rise to be controlled, and such change in the irradiation technique may therefore provide the opportunity of raising the dose above 4 5 Mrad without the necessity of using types of plastic which possess greater resistance

SUMMARY

The radiation resistance has been determined on two strains of *Streptococcus faecium* and on spores of two strains of *Bacillus subtilis* dried in atmospheric air after suspension in various media and storage for various periods In addition, the radiation resistance of the spores was determined before and after cleaning and after culture at 45° C

Resistance to ionizing radiation of *Str faecium* as well as subtilis

spores were found to be highest if they were dried in a protein-containing milieu Unwashed spores had a greater resistance than washed spores Culture at higher temperature and heat activation at 80° C for 10 minutes had no influence on the resistance of the subtilis spores

In relation to the present study, it is recommended that doses in radiation sterilization should be indicated as minimum doses and, if equality is to be established between the effect of radiation sterilization and the effect of heat sterilization, it should be based on the maximum resistance of the most resistant ones of the commonly occurring micro-organisms, under the conditions prevailing during the given sterilization procedure

A dose of 2 5 Mrad is used at several centres as a routine dose for sterilization of medical equipment This dose gave an inactivation factor of only 10^2 to 10^3 for the faecium strains studied, when the strains were dried in serum broth This factor is very low in comparison with the effect of autoclaving under the conditions usually recommended At Risø the Research Establishment of the Danish Atomic Energy Commission, the sterilizing dose for disposable items of hospital equipment is at present 4 5 Mrad

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17 *Powers E L* *Webb R B* & *Ehret C F* Storage transfer and utilization of energy from X rays in dry bacterial spores Radiation Res Suppl 2 94 1960
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Acta path et microbiol scandinav 62 459–460 1964

BRIEF REPORT

REGENERATION OF PARIETAL PERITONEUM

By *Gunnar Eskeland*

Reconstitution of mesothelial defects has been considered to take place

a) from intact mesothelium surrounding the wound (3),
b) from mesothelial cells detached from peritoneum and implanted on the wound as free grafts (2)
c) by metaplasia of cells in the connective tissue underlying the wound (1) and
d) by a combination of these mechanisms (4)

This report deals with an investigation in which the following experiments were performed

1 Wounds were made in the parietal peritoneum of rats either by burning at 59° C for 15 seconds or by the excision of circular areas including peritoneum the underlying connective tissue and part of the muscle The wounds were of three different sizes with diameters 4 mm 12 mm and 36 mm After periods varying from 1 hour to 14 days the abdominal wall carrying the wounds was excised The peritoneal surface was treated with a weak silver nitrate solution after which the

alcohol Hautchen pre

Efskind (3) and were

ixed in buffered OsO_4

... embedded in Vestopal W Sections for light and electron microscopy were cut on a Huxley microtome

2 Rats in which excision wounds had been made were injected with colcemid (Ciba Basle) *1 mg/200 g body* weight 4 hours prior to sacrifice The abdominal wall carrying the wounds was excised 1 8 days after the wounds had been made and Hautchen preparations were produced as in the *previous experiment*

3 Small plastic discs made of Vestopal W were placed in the scrotal cavities of rats in wh h th ... minal cavity

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slides

Results

1 As early as 1 and 4 hours after the wounds had been made large numbers of cells were observed resting on a fibrin scaffolding and more or less completely covering the surface of the wound These cells were of the same appearance as the free peritoneal fluid cells (the monocyte/macrophage type and were present before

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Received 26 ix 64 *from Universitetet i Oslo Institutt for Patologisk Anatomi Rikshospitalet Oslo* (Head Professor *Olav Torgersen* M D)

This investigation was supported by grants from *The Norwegian Cancer Society* and from *Doktor Alexander Malthes Legat*

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BRIEF REPORT

PHOTOINACTIVATION OF VISNA VIRUS

By *Halldor Thormar*[1] and *Inger Petersen*

Viruses show a marked variation in their sensitivity to visible light applied in the presence of photodynamic dyes and it has been suggested that the rate of photo dynamic inactivation of a virus is a characteristic property which might be useful for taxonomic classification of newly isolated animal viruses (1 2)

In an attempt to characterize visna virus a recently isolated virus of sheep (3) a number of its physical chemical and biological properties has been studied in cluding its sensitivity to visible light in the presence of toluidine blue The virus was grown in cultures of sheep cells in medium 199 with 1 per cent sheep serum (4) and was diluted tenfold in phosphate buffered saline (without Ca and Mg) pH 7 3 The irradiation procedure was similar to that employed by *Hiatt et al* (1) Toluidine blue (Merck) was added to the virus in a final concentration of 6 µg per ml (2×10-5 M) and the mixture was kept in the dark at 4° C for 45 minutes Twenty five ml of the mixture were then poured into an aluminium dish 65 mm in diameter and irradiated with a 250 W Osram Nitraphot SR lamp at a distance of 90 cm from the bottom of the dish During irradiation care was taken to secure an adequate stirring and temperature control of the fluid Samples of 0 3 ml were taken at intervals, diluted tenfold in Hanks solution and dialysed in the dark against two changes of Hank s solution before titration of surviving virus

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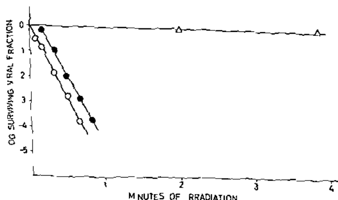


Fig 1

Received 31 x 64 from the *Enterovirus Department Statens Seruminstitut* Copenhagen

[1] Present address Institute for Experimental Pathology University of Iceland Keldur Reykjavik

an appreciable cellular infiltration could be demonstrated in the tissues underlying the wound None of the cells observed within the limits of the defect at these early stages resembled mesothelial cells

From 2 days onward single cells or islands of cells with the characteristics of mesothelium were found scattered over the wound surface These cell islands continuously increased in size until even the largest wounds were completely covered with mesothelium at 8 10 days

2 In rats treated with colcemid *numerous mitoses were found both in the mesothelial like cells* scattered over the wound surface and in a narrow brim of intact mesothelium bordering on the wound In both locations mitoses were numerous at 2 days and maximum mitotic activity was recorded 3 and 4 days after the wounds were made

3 The plastic discs were partly or completely covered with cells in one or several layers *The superficial layer was composed of flattened cells which in many in* stances formed continuous sheets with the characteristics of mesothelium

4 The central filters of the diffusion chambers were partly or completely covered by cells which were of two main types Typical fibroblasts and cells with the same appearance as proliferating mesothelium seen in healing peritoneal wounds

Conclusions

Free mononuclear cells from the peritoneal fluid may become implanted on to the surface of peritoneal wounds and have the potentialities of differentiating to form mesothelial cells These cells multiply and are considered to be a major source of the mesothelial cells ultimately covering the defect

The intact mesothelium in the immediate vicinity of the wound also participate in the repair process by proliferation and ingrowth

Detailed reports of the experiments will be published

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TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting September 26, 1964

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SYMPOSIUM ON MODERN METHODS IN ROUTINE HISTO-PATHOLOGY

S Falkmer HISTOCHEMISTRY

J Ahlqvist ENZYME HISTOCHEMISTRY

The mechanisms of the metal the azo dye the indigogenic and the tetrazolium techniques for demonstrating enzymes in tissue sections were described Some of the difficulties in the interpretation of results were discussed amongst others those caused by uneven distribution of substrates in the tissues This makes quantitation of reactions difficult as far as regards the number of reactive sites It does not, however constitute a drawback for enzyme histochemistry since the forces resulting in the uneven distribution may be the same as those leading to the formation of the enzyme substrate complex at least in some cases The uneven distribution of sub strates thus might include part of the mechanism of enzyme activity Certain hitherto obscure findings in enzyme chemistry might be explained if the local concentration of substrate accessible to the action of reactive sites sometimes were dependent on structures other than the molecule carrying the reactive site With regard to the function of enzymes *in vivo* this would imply that it sometimes might be preferable to carry out enzyme studies on as intact tissues as possible

L Esposti CYTOCHEMICAL STUDIES ON PROSTATIC CARCINOMA

G Moberger SULPHYDRYL GROUPS AND CANCER

A Ljungqvist JUXTAGLOMERULAR GRANULES

B Engfeldt X RAY METHODS

A Bergstrand ELECTRON MICROSCOPY

F Linell THE DIAGNOSIS OF PARASITIC DISEASES

B F rs STAINING OF FUNGI

G Nathorst Windahl STAINING OF PNEUMOCYSTIS

During the last few years endemics and isolated cases of Pneumocystis pneumonia have occurred in Sweden and 20 deaths have been recorded

of visna virus was almost identical to that of vaccinia virus 90 per cent of the infectivity being lost in about 10 seconds Poliovirus was *not significantly inactivated* by irradiation for 4 minutes Preincubation with toluidine blue in the dark at 4° C for 2½ hours instead of 45 minutes before irradiation did not cause a more rapid inactivation of visna virus Incubation with dye in the dark throughout the experimental period did not cause a significant inactivation of either visna or vaccinia virus

The present experiment shows that visna virus is relatively sensitive to photodynamic inactivation under the conditions employed and equally sensitive as vaccinia virus Poliovirus on the other hand was stable and has recently been found to require other conditions in order to be inactivated (8) The reason for the differential sensitivity of viruses to photodynamic action is not known *Hiatt* (2) has suggested that it reflects differences in permeability of the viral protein coat to penetration of dye *The visna virus particles are rather incompact being coated by a thin* membrane apparently derived from the cytoplasmic membrane of the host cell (5) Vital dyes like toluidine blue may therefore have an easy access to nucleic acid in the interior of the virus explaining that maximal rate of photodynamic inactivation is observed after preincubation of virus and dye in the dark for 45 minutes or less at 4° C, pH 7 3 The size and external structure of visna virus is apparently similar to that of influenza virus and avian and mouse tumour viruses (5 6) *Photoinactivation* of influenza virus has been reported recently under conditions somewhat different from those employed in the present study (7 9)

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For the identification of the implicated organism Pneumocystis carinii the following stains are recommended: PAS-gallocyanin-chromalun staining and either a STB method (sulphation toluidine blue method) or a modification of Gomori's methenamine silver nitrate procedure. The STB stain used is a modification of the procedure described by *Chalvardjian & Grawe*. The sections are deparaffinized in xylene, which is washed off in ether, and then placed in a sulphuric acid-ether reagent at + 4° C for 15 minutes. After washing in water the slides are placed in 0.06 per cent toluidine blue (Hoyer-Merck) for 15 minutes, washed in water, dehydrated in isopropylalcohol, cleared in xylene and mounted.

With the PAS-gallocyanin-chromalun staining the membranes of all cyst forms stain red and the internal structures grey-blue. The STB procedure stains thick-walled cysts metachromatically violet; with the methenamine-silver nitrate technique the thick-walled parasites stain grey-black.

For smears the Giemsa method and the STB procedure are recommended. With the Giemsa stain the internal structures of the parasites are visualized, whereas the STB method stains the membranes.

G. Moberger: FINE NEEDLE ASPIRATION BIOPSY

S. Franzén: FINE NEEDLE ASPIRATION BIOPSIES IN THE NECK REGION

S. Dahlgren: INTRA-THORACIC NEEDLE ASPIRATION BIOPSIES

J. Pontén: TISSUE CULTURE

J. Mellgren: TISSUE CULTURE ON CARCINOMA IN SITU AND INVASIVE CARCINOMAS OF THE CERVIX

B. Stenkvist: BENIGN AND MALIGNANT FIBROBLASTS *IN VITRO*

Acta path et microbiol scandinav 62 465 471 1964

Oslo University Institute of Pathological Anatomy Rikshospitalet Oslo Norway
Prof O Torgersen MD) Histochemical Laboratory (S D Schultz Haudt PhD)

COMPARATIVE HISTOCHEMISTRY OF NON INJURED SKIN CONNECTIVE TISSUE OF NORMAL AND ASCORBIC ACID DEFICIENT GUINEA PIGS

By

STIG D SCHULTZ HAUDT and SIGURD HJ FROM

Received 29 ii 64

The relation of ascorbic acid to connective tissue metabolism has been the subject of much study particularly with respect to the function of vitamin C in the biosynthesis and maintenance of collagen (*Gould* 1963) There are however, also many reports describing alterations in ground substance polysaccharides in ascorbic acid deficiency Mostly these have been concerned with changes occurring in healing wounds or carrageenin induced granulomas in normal as compared to vitamin C deficient guinea pigs and the observations made have been conflicting (*Gould* 1963)

The investigations here presented represent efforts to establish quantitative differences in the polysaccharide contents of non injured skin connective tissues of normal and vitamin C deficient animals by a combination of histo and biochemical techniques

MATERIALS AND METHODS

Young guinea pigs weighing 250 g were used in the experiments Some of the animals were maintained on an ascorbic acid deficient diet until they had suffered considerable losses in body weight usually for 18 to 20 days These and control animals were then treated in an identical manner They were anaesthesized by ether inhalation and the hairs of the trunk were clipped whereafter the animals were sacrificed
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Portions of the dried extracts were then subjected to paper electrophoresis on Whatman no 3 MM
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This investigation was supported by Grant no DE 01472 03 from the NIDR of the National Institutes of Health Bethesda Maryland U S A

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Fig 1

The figure represents a photograph of stained paper strips after electrophoresis of skin extracts from normal and vitamin C deficient guinea pigs The two upper strips are stained with toluidine blue the two lower by means of PAS The first and third strips from the top represent the results obtained with normal skin extract, the second and fourth those with the extract from ascorbic acid deficient animals The component with the greatest mobility towards the anode (+) contained chondroitin sulphates the slower moving one hyaluronate, whereas the slowest migrating component represents glycoprotein In all cases identical amounts of the extracts had been applied to the paper strips prior to electrophoresis

was found not to be confined to any one particular type Following incubation with the hyaluronidase, a zone representing chondroitin sulphate B showed up after electrophoresis and staining, although the colour intensity of the zone was fainter than with identical amounts of undigested extracts

With regard to glycoproteins as demonstrable by means of PAS, it appeared that there were considerably smaller amounts of such materials present in normal skin extracts (the second strip from the bottom in Fig 1) than in identical amounts of extract from vitamin C deficient animals (the bottom strip)

The impressions gained from the paper electrophoresis experiments were confirmed by the chemical analyses The results of these are shown in Fig 2

As seen from Fig 2, there was slightly more total acid mucopolysaccharide material present in the extract of normal skin than in that of the skins of the ascorbic acid deficient animals Under normal conditions, there were 1 7 times as much chondroitin sulphate as hyaluronate (in terms of galactosamine and glucosamine, respectively) In ascorbic acid deficiency on the other hand, there was twice as much hyaluronate present as chondroitin sulphate With regard to the heteropolysaccharides of the glycoproteins, the extracts from the vitamin C deficient animals contained nearly four times as much as did those from normal animals

Thus the results, showed that in ascorbic acid deficiency, there is more ground substance polysaccharide material present in the skin connective tissue than under normal conditions This increase is due to an accumulation of "neutral heteropolysaccharide" containing materials, *i e* glycoproteins In vitamin C deficiency, there is also more hyaluronic acid present than under normal conditions The total

Identical amounts of dried extracts were exposed to testicular hyaluronidase (Wydase Wyeth Inc)
M acetate buffer of pH
reducing units of the e
running tap water, lyophilized and subjected to paper electrophoresis as above

Following electrophoresis the paper strips were dried at 100° C and one of each series divided lengthwise into two 2 cm wide ribbons One of these was stained with toluidine blue in order to locate hyaluronic acid and chondroitin sulphuric acids (*Schultz Haudt et al* 1964), whereas the other was stained by the periodic acid Schiff technique (PAS) as described by *Aronsson* (1961) for the localization of glycoproteins Areas corresponding to the stained zones on the ribbons were cut out from the remaining, unstained strip and eluted with water The eluates were dialysed and subsequently lyophilized

The amounts of glycoprotein present in the eluates were determined by the colourimetric PAS method of *Hooghwinkel & Smits* (1957)

Dried eluates from the zones corresponding to those that stained with toluidine blue were hydrolysed with 2 ml of 6 N HCl for 4 hours at 110° C in sealed test tubes The hydrolysates were treated with charcoal filtered and evaporated to dryness by distillation at reduced pressure and a temperature of 40° C They were next redissolved in water, and the solution added onto a column (0 9 by 10 cm) of a cationic exchange resin (Dowex 50 H+, X-12) Hexosamines were eluted from the column with 0 5 N HCl 10 ml being collected The eluates were lyophilized They were finally redissolved in appropriate amounts of water and subjected to paper chromatography (*Stoffyn & Jeanloz* 1954) and a colourimetric reaction (*Roseman & Daffner* 1956) in order to determine the presence and amounts of glucosamine (representative of hyaluronate) and of galactosamine (representative of chondroitin sulphates)

The incubation with hyaluronidase was carried out for control purposes as well as in order to differentiate between chondroitin sulphate B and the A and/or C varieties of this acid mucopolysaccharide The enzyme preparation used catalysed the hydrolysis of hyaluronate as well as that of chondroitin sulphates A and C but not that of type B

RESULTS

Typical, stained electrograms are shown in Fig 1 As seen in the figure, the extracts from both normal and ascorbic acid deficient animals divided upon electrophoresis into 3 polysaccharide containing components, all migrating towards the anode The slowest migrating one stained by PAS only (the two lower strips) and, hence, contained heteropolysaccharide material characteristic of glycoproteins, but different from the acid mucopolysaccharides (*Schultz-Haudt et al* 1964) The two faster migrating components reacted with toluidine blue only (the two upper strips in Fig 1) The slowest of the two contained glucosamine and disappeared after incubation of the extract with hyaluronidase It, therefore, was judged to contain hyaluronic acid The fastest moving one contained galactosamine, but was only partially susceptible to the action of the enzyme Hence, this component was considered to contain a mixture of chondroitin sulphates C (and or A) and of B (resistant to the enzyme preparation) By visual comparisons of the strips—onto which identical amounts of the extracts had been applied—it appeared that normal skin (the strip on top of Fig 1) contained considerably more of chondroitin sulphates than of hyaluronate This situation appeared reversed in vitamin C deficiency (the second strip from the top) In similar experiments, the reduction in the contents of chondroitin sulphates in the ascorbic acid deficient animals

Firstly, since most of the earlier investigations were based on studies of healing wounds of the carrageenin induced granuloma, or of tissue growth in polyvinyl sponge implants Secondly, because many previous studies have been strictly histochemical in nature, and evaluations of colour intensities in tissue sections as observed in the light microscope are uncertain except in extreme cases Thirdly, because there has in the past been uncertainties about the chemical basis for some of the methods used in connective tissue polysaccharide histochemistry

With regard to the latter point the present as well as other investigations (*Schultz Haudt et al* 1964) have demonstrated that so far as skin and mucous membranes are concerned a differentiation between glycoproteins (containing the so-called "neutral heteropolysaccharides) and mucoproteins (containing acid mucopolysaccharides) may be made by means of the PAS technique and the toluidine blue stain, respectively

In relation to the second point the method used in the present experiments permits for fairly satisfactory quantitative comparisons Moreover, a distinction may be made between types of chondroitin sulphates

Taking the above considerations into account, the present findings are in agreement with the previous suggestions made (*Gersh & Catchpole* 1949 1960, *Persson* 1953) to the extent that there is an increase in ground substance in scurvy This increase, however, is due to an accumulation of glycoprotein material With regard to total mucopolysaccharide—in terms of hyaluronate and chondroitin sulphates—a slight decrease was found in vitamin C deficiency This is in conflict with some earlier observations (*Bunting & White* 1950 *Bradfield & Kodicek* 1951 *Persson* 1953 *Dunphy et al* 1956 *Robertson & Hinds* 1956 *Kimoto et al* 1959) The apparent disagreement is however, at least in part, resolvable by considering the two types of acid mucopolysaccharides under consideration separately As shown by the present results, there is an increase in hyaluronic acid in ascorbic acid deficiency, as compared with the normal situation This is in agreement with observations made on healing scorbutic wounds (*Gould* 1963) and on the carrageenin induced granuloma (*Slack* 1957 1958) On the other hand, there is a marked decrease in the amounts of chondroitin sulphates in vitamin C deficiency accounting for the slight reduction in total acid mucopolysaccharide That reduced amounts of chondroitin sulphates are present in scurvy as compared to normal conditions is in accordance with the conclusions arrived at by *Kodicek & Loewi* (1955) with respect to healing tendons by *Slack* (1958) in his studies of the carrageenin granuloma, by *Stein & Wolman* (1958) in their investigations of healing wounds, and by *Hughes & Kodicek* (1960) experimenting with granulation tissue

It has been suggested that the ground substance polysaccharides in scurvy are depolymerized and that they are more soluble than under normal conditions (*Gersh & Catchpole* 1949 *Persson* 1953) In the pre-

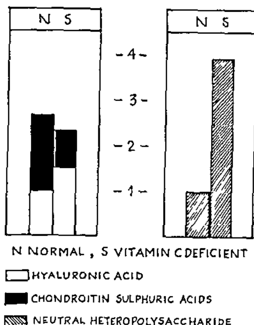


Fig 2

A schematic illustration of the results obtained with respect to the amounts of the examined polysaccharides present in extracts from normal and vitamin C deficient guinea pig skin The unit is selected at random and does not refer to amounts present on for example a dry weight basis Hence in terms of the total amounts of the types of polysaccharides present the two halves of the figure are not comparable Total acid mucopolysaccharide was arrived at by adding the measured amounts in eluates from the paper electrophoresis strips of glucosamine (= hyaluronic acid) and galactosamine (= chondroitin sulphuric acids) In spite of the increase in hyaluronic acid in ascorbic acid deficiency there was a slight decrease in total acid mucopolysaccharide due to the reduction in the amounts of chondroitin sulphuric acids —The colourimetric measurements of neutral heteropolysaccharides showed a great increase of such material in vitamin C deficiency An absolute quantitation was impossible due to the lack of suitable reference substances

amounts of acid mucopolysaccharides present in ascorbic acid deficiency, however, are somewhat reduced as compared with normal animals due to a considerable reduction in chondroitin sulphates

For control purposes, the collagen contents of skin homogenates as well as of the extracts from the two groups of animals were determined in terms of the contents of hydroxyproline following hydrolysis (*Leach* 1960) The much higher amounts of this amino acid present in the materials from the normal animals were considered indicative of true ascorbic acid deficiency in the experimental group of guinea pigs

DISCUSSION

The results here presented show that ascorbic acid deficiency causes definite alterations in the polysaccharide composition of the ground substance of othewise non-injured guinea pig skin connective tissue The results, however, appear not entirely comparable to most of the observations made previously by other investigators, for several reasons

Firstly, since most of the earlier investigations were based on studies of healing wounds, of the carrageenin induced granuloma, or of tissue growth in polyvinyl sponge implants. Secondly, because many previous studies have been strictly histochemical in nature, and evaluations of colour intensities in tissue sections as observed in the light microscope are uncertain except in extreme cases. Thirdly, because there has in the past been uncertainties about the chemical basis for some of the methods used in connective tissue polysaccharide histochemistry.

With regard to the latter point, the present as well as other investigations (*Schultz Haudt et al* 1964) have demonstrated that so far as skin and mucous membranes are concerned, a differentiation between glycoproteins (containing the so-called "neutral heteropolysaccharides") and mucoproteins (containing acid mucopolysaccharides) may be made by means of the PAS technique and the toluidine blue stain, respectively.

In relation to the second point, the method used in the present experiments permits for fairly satisfactory quantitative comparisons. Moreover, a distinction may be made between types of chondroitin sulphates.

Taking the above considerations into account, the present findings are in agreement with the previous suggestions made (*Gersh & Catchpole* 1949, 1960, *Persson* 1953) to the extent that there is an increase in ground substance in scurvy. This increase, however, is due to an accumulation of glycoprotein material. With regard to total mucopolysaccharide—in terms of hyaluronate and chondroitin sulphates—a slight decrease was found in vitamin C deficiency. This is in conflict with some earlier observations (*Bunting & White* 1950, *Bradfield & Kodicek* 1951, *Persson* 1953, *Dunphy et al* 1956, *Robertson & Hinds* 1956, *Kimoto et al* 1959). The apparent disagreement is, however, at least in part, resolvable by considering the two types of acid mucopolysaccharides under consideration separately. As shown by the present results, there is an increase in hyaluronic acid in ascorbic acid deficiency, as compared with the normal situation. This is in agreement with observations made on healing scorbutic wounds (*Gould* 1963) and on the carrageenin induced granuloma (*Slack* 1957, 1958). On the other hand, there is a marked decrease in the amounts of chondroitin sulphates in vitamin C deficiency, accounting for the slight reduction in total acid mucopolysaccharide. That reduced amounts of chondroitin sulphates are present in scurvy as compared to normal conditions is in accordance with the conclusions arrived at by *Kodicek & Loewi* (1955) with respect to healing tendons, by *Slack* (1958) in his studies of the carrageenin granuloma, by *Stein & Wolman* (1958) in their investigations of healing wounds, and by *Hughes & Kodicek* (1960) experimenting with granulation tissue.

It has been suggested that the ground substance polysaccharides in scurvy are depolymerized and that they are more soluble than under normal conditions (*Gersh & Catchpole* 1949, *Persson* 1953). In the pre-

sent experiments, no efforts were made to evaluate these suggestions However, the respective rates of migration of the various polysaccharide components upon electrophoresis were identical whether they derived from ascorbic acid deficient animals or not The possibility of increased solubilities in scurvy may be thought to be reflected in the obtained data with regard to hyaluronic acid and glycoproteins As a control, tissue residues after NaCl extraction were further extracted with 0 5 N NaOH, and the extracts were analysed for the presence of hexose and hexosamine The analyses showed that the NaCl extraction had not been entirely exhaustive in terms of removing all sugar containing components On the other hand, the amounts of such materials left over were not sufficiently large to significantly influence the results presented

An interpretation of the observations made, in terms of differences in tissue biochemistry, is difficult It seems, however, reasonable to suppose that when it comes to the acid mucopolysaccharides, lack of ascorbic acid interferes with the synthesis by the cells of chondroitin sulphates This interference may be related to a reduced rate of sulphation of precursors (*Kodicek & Loewi* 1955, *Stein & Wolman* 1958) although it is a matter of some dispute whether or not the rate of sulphation is affected by ascorbic acid (*Friberg* 1958, *Robertson* 1961) More probably, there is a reduction in the rate of synthesis of the chondroitin sulphates in general (*Slack* 1957, 1958) How this is related to an increased rate of synthesis—or maybe a retarded rate of breakdown—of hyaluronic acid is, at the moment, hard to evaluate

The excessive accumulation of glycoproteins in vitamin C deficiency may not be primarily related to tissue metabolism It is suggested that it may, at least in part, be the result of an increased infiltration of the connective tissue by serum glycoproteins because of the injury to the capillaries associated with ascorbic acid deficiency A suggestion of the kind is in disagreement with *Pirani & Catchpole* (1951) who observed that the serum glycoprotein concentration increased in scurvy, but felt that this was due to the absorption into the circulation of depolymerized connective tissue polysaccharides

SUMMARY

Ground substance polysaccharides of skin connective tissue of normal and vitamin C deficient guinea pigs have been studied by means of a combination of histo and biochemical techniques

As compared to the normal tissue, the skins from the ascorbic acid deficient animals contained

1 considerably more polysaccharide materials This was found to be due to an accumulation of heteropolysaccharide material characteristic of glycoproteins,

2 slightly less total acid mucopolysaccharide material This was due to a sharp reduction in the amounts of chondroitin sulphates present,

3 more hyaluronic acid, although not enough to compensate for the reduction of chondroitin sulphates in terms of the total amounts of acid mucopolysaccharides present

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MASSIVE FATAL PNEUMOCYSTIS PNEUMONIA IN LEUKAEMIA

Report of Two Cases

By

G NATHORST-WINDAHL, B H HESSELMAN, B SJOSTROM and JAN PONTÉN

Received 18 ii 64

Pneumocystis pneumonia (Pcp) manifests itself as an interstitial pneumonia, often characterized by a plasmacellular reaction This pulmonary disease has been known in Europe for more than two decades as "interstitial plasma cell pneumonia" In infants it is a serious, often epidemic, disease carrying a high mortality Pcp is uncommon in older age groups where it ordinarily is of a benign character and usually is detected at autopsy as small circumscribed pneumonias (*Mur* 1959) Massive fatal Pcp in older children and adults is rare

Pcp and the aetiological rôle and biological nature of the implicated organism Pneumocystis Carinii (PC) have been reviewed (*Jirovec & Vanek, Carlgren & Nathorst-Windahl* 1955, *Hamperl* 1956, *Gajdusek* 1957, *Jirovec* 1959)

The first case to be described is one of a four year-old girl with acute leukaemia treated with adrenocorticoids and cytostatics for several months The leukaemia was in remission at death and the immediate cause of death was respiratory insufficiency due to a massive Pcp The second case is one of a 55-year old man with lymphatic leukaemia of eight years duration The leukaemia was treated with corticosteroids and cytostatics In this case the immediate cause of death was also a massive Pcp Both cases occurred in the same hospital but in different wards

The cases seem to be of interest not only because of their rarity (they are in fact the first cases of Pcp in older age groups described in Swe den) but also as an illustration of a possible connection between pro longed treatment with corticosteroids and cytostatics and the develop ment of Pcp

CASE REPORTS

Case 1 The patient was a girl born in September 1954 who was admitted to the hospital in August 1958 with a history of a sore throat and fever Penicillin had been



Fig 1

Case 1 Pneumocystis pneumonia The alve li contain characteristic foamy material Moderate interstitial broadening and infiltration with chiefly lymphocytes PAS gallocyanine × 160

given for a few days At admission the child was acutely ill with high fever and

al to t normal The bone marrow smear examination was rendered difficult by a technically unsatisfactory puncture but the picture strenghtened the suspicion of leukaemia However as a remission seemed to have ensued the patient was discharged two weeks after admission but asked to meet f r ambulatory follow up The

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Figs 2 and 3

Fig 2 Case 1 Pneumocystis pneumonia Honeycombed appearance of alveolar content typical of Pneumocystis Carinii infection PAS gallocyanine × 385

Fig 3 Case 1 Lung Alveolar content with Gram positive Pneumocystis Carinii organisms Gram Weigert × 700

During the following months the course was satisfactory but subsequently a fever and continuous dyspnoea developed The latter was considered due to a disturbance in the electrolyte equilibrium in connection with the intense protracted corticosteroid treatment The total quantity of prednisolone and mercaptopurine given was 3870 mg and 5000 mg respectively The prednisolone was now replaced by dexamethasone (Decadron MSD) However the child's condition became worse and she was re admitted for the last time in May 1959 The patient was now gravely ill and had a rapid laborious and shallow respiration X ray examination of the lungs showed widespread small patchy infiltrations and milky shadows on both sides A disseminated pulmonary candidiasis was suspected since the patient had previously had an oral moniliasis Mycostatin (Bofors) was administered without effect Oxygen inhalation had but very slight beneficial effect Respiratory difficulty and dyspnoea increased and three days after the last admission the patient died

Pathologic Findings

Lungs Grossly the tissue was diffusely consolidated and grey in formol fixed state The cut surface showed a distinct lobulation Lighter polygonal areas of a maximal diameter of about 8 mm were surrounded by darker broadened septa

Microscopically the diffuse character of the process could be confirmed and no normal areas were found in a large number of sections The majority of the alveoli were filled by honeycombed faintly eosinophilic masses which were often retracted from the alveolar walls (Figs 1 2) In some areas the alveolar content was more compact and partly had the appearance of hyaline membranes lining the alveoli A few bronchioli were also filled by honeycombed material Gram Weigert stainings showed that this material consisted of large numbers of intraalveolar Gram positive rounded or crescent like forms of the parasite (Fig 3) Their sizes were 5–7 my With the silver methenamine method also numerous organisms were seen PAS gallocyanine stain showed an intense positive reaction in the mucoid envelopes of the parasites

In such alveoli as were filled by the honeycombed P C colonies the lining cells were cuboid flattened and had partly desquamated Other alveoli in which the

eosinophilic material was more sparse had prominent lining cells with vesicular nuclei containing nucleoli Such cells were often desquamated and were lying free in the alveolar lumina The alveolar septa were often broadened and infiltrated by inflammatory cells Most of these cells were lymphocytes but granulocytes some of which were eosinophilic and histiocytes were also seen

With methyl green pyronine stain only small numbers of plasma cells were seen The inflammatory cells were most numerous in the septa and around blood vessels The interlobular septa were broadened by oedema No intranuclear inclusion bodies were seen

Sudan IV stain showed areas with macrophages and desquamated alveolar cells

sis These cells were interpreted as blast cells belonging to the myeloic series

Adrenal glands Microscopic investigation showed an atrophic cortex with narrow fasciculate and reticular zones Sudan IV stain showed a marked reduction of lipid content especially in the zones mentioned

Case 2 A man born in July 1905 chronic lymphocytic leukaemia had persisted since 1952 He had been working until 1956 at which time his superficial lymph n les increased in size At this time he received treatment with local irradiation There was a m derate regression of the lymph node enlargement and the white blood cell count decreased from 332000 to 75000 per cu mm

In March 1959 the patient was admitted to the hospital for an acute otitis media with mastoiditis and purulent meningitis Mastoidectomy and intense antibiotic therap

owed bilateral pleurisy There

In January 1960 an X ray examination

and the patient received irradiation to the

a moderate fever and X ray of the chest

filtrati ns in the right lung radiating from the hilar region (Fig 4) The co

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there was no effect of either antibiotics or steroids given in large doses

In November 1959 and July 1960 the relative values for gamma globulin in blood were 6 5 and 5 4 per cent respectively which indicated a grave hypogammaglobulinaemia A week before death the patient received 16 ml of gamma globulin without any effect n the respiratory distress

Terminally the patient had a shallow respiration and cyanosis and he died in respirat ry insufficiency in July 1960

Autopsy revealed heavy and consolidated lungs with a greyish yellow and firm parenchyma The pleura was of n rmal appearance The liver weighed 2500 g and had a h m gen us pale

Hist l gical examinati

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The periodic acid Schiff gallo cyanine stain c loured the alveolar content very distinctly with PAS positive mucoid envel pes of the parasites and in many of the envelopes small dots and bars of gallocyanine positive nuclear material With Gram Weigert stain isolated cyst or



Fig 4

Case 2 Roentgenogram of chest March 1960 Rather dense localized infiltrations in the right lung radiating from the hilar region



Fig 5

Case 2 Roentgenogram of chest April 1960 Rather massive streaky infiltrations in both lungs radiating from the hilar regions



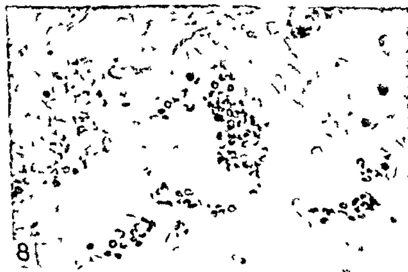
Fig 6

Case 2 Roentgenogram of chest July 1960 Streaky and nodular confluent infiltrations in the major part of the right lung Prominent infiltrations also in the left lung



Fig

Case 2 Pneumocystis pneumonia All al e li in the field contain honeycoml ed colon es of Pneum cystis Carinii Interstit al infiltration ch efly l y lymphl ocytes I AS galloc an ne × 140



F g 8

Ca e 2 Lung Many silver impregnate l Pnet m cyst s Carinii organisms are seen as rings r crescents Silver methenam ne × 420

crescent formed Gram positive parasites were clearly demonstrated and with the silver methenamine m th [illegible]
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leukaemic infiltrations with small accumulations of mature lymphocytes in the portal tracts in the liver and in the spleen, a rather diffuse infiltration with lymphocytes

DISCUSSION

The diagnosis of leukaemia and Pcp seems to be established beyond doubt in both cases

In the first case the clinical picture was typical of an acute subleukaemic leukaemia and two sternal marrow biopsies contained large numbers of small blast cells interpreted as paramyeloblasts The leukaemia was evidently in (partial) remission at death and no blast cells were noted in the liver at autopsy and found only in moderate numbers in the spleen

In the second case the diagnosis of leukaemia was based on a positive sternal marrow biopsy and on the white cell count of 332000 per cu mm on one occasion with dominance of mature lymphocytes There was also enlargement of superficial and mediastinal lymph nodes, the liver and the spleen At autopsy lymphocytic leukaemic infiltrations were found in the liver and spleen and, to a lesser degree, in the lungs

Pcp was not diagnosed *intra vitam* but the histological demonstration of a honeycombed eosinophilic alveolar content with large numbers of P C in the lung section was unequivocal

In spite of the fact that the parasite P C has been known since 1910 and thoroughly investigated with histological and histochemical methods (*Carini* 1916, *Opferkuch* 1959) and with the electron microscope (*Seifert* 1960, *Seifert & Pliess* 1960) the taxonomic state of the parasite has not been fully elucidated The organism is usually considered to be a protozoon and has not yet been cultured *in vitro*

Cultures from the lungs were not attempted in the present cases

The inflammatory reaction of Pcp in infants is usually characterized by the presence of numerous plasma cells Our cases differ in this respect since plasma cells were found only in small numbers This may well be due to the radiation therapy in the second case and to the prolonged steroid treatment in both cases, which factors are known to depress plasmacytogenesis and production of antibody Case 2 also showed a pronounced hypogammaglobulinaemia thus providing interesting analogies to the reports of Pcp in infants with agammaglobulinaemia (*Kramer* 1962)

In retrospect the respiratory distress during the last days of life in both of our cases taken together with the roentgenological lung picture

and the insignificant physical findings in the lungs are typical clinical signs of Pcp (*Eberling & Cohen* 1958, *Schafer* 1955)

Whether the combination of leukaemia and Pcp was only fortuitous or whether a pathogenetic connection existed is of great interest Recent observations indicate that serious diseases affecting the reticuloendothelial system may lessen the resistance against P C Circumscribed Pcp has thus been diagnosed at autopsy in adults with Hodgkin's disease (*Mur* 1959, *Vanek* 1953) and various forms of leukaemia (*Hendry & Patrick* 1962, *Jirovec & Vanek* 1954)

In addition to the possible rôle played by leukaemia *per se* in preparing the ground for Pcp, the intense treatment with corticosteroids, antibiotics and cytostatics was conceivably of importance (*Kossel* 1962, *Muller* 1960, *Roos & Keller* 1960)

It is well known that corticosteroids, especially when combined with antibiotics, lessen the resistance against infection, and evidence of a connection between administration of adrenocorticoids combined with antibiotics and Pcp has been derived from experiments with rats (*Pliess & Trode* 1958, *Weller* 1955 and 1956) If rats are treated with antibiotics and corticosteroids during a long period of time Pcp will often develop These observations have been interpreted as an indication that P C normally exists as an innocent commensal in the lungs of the rat When the resistance is diminished as a result of treatment, P C will be able to grow unimpeded and attain a pathogenic character By analogy the same mechanism may well be of importance in explaining the unusual occurrence of a massive Pcp, terminating fatally in our patients Massive administration of steroids is frequently used in current therapy On the basis of an ubiquitous occurrence of P C one would therefore expect massive Pcp to be common in such cases Since that is not the case additional, unknown pathogenic factors may also be operative

In a review of Pcp in association with prolonged corticosteroid therapy in older children *Kossel* (1962) pointed to the fact the Pcp cases occurred in connection with viral "grippaler infekt ketten" Although there was no direct proof of a coexisting virus infection in our cases this possibility cannot be wholly eliminated

In this connection the frequent coexistance of Pcp and cytomegalic inclusion body disease may be mentioned (*Hamperl* 1956, *Kramer* 1962, *Symmers* 1960), signs of the latter disease, however, were not found in our cases

SUMMARY

Two cases of Pneumocystis pneumonia are presented, one in a subleukaemic acute leukaemia in a four-year-old girl and one in chronic lymphatic leukaemia in a 55-year-old man The patients were treated with large doses of corticosteroid for prolonged periods, periodically

crescent-formed Gram positive parasites were clearly demonstrated and with the *silver methenamine method* numerous organisms were also seen (Fig 8) There was a moderate interstitial diffuse fibrosis and here and there broadening of alveolar walls and a moderate infiltration by lymphocytes, histiocytes and some granulocytes In methyl green pyronine stain only a very small number of plasma cells were seen There was only a slight leukaemic infiltration, in places with small accumulations of lymphocytes in and around blood vessels

Examination of the *liver* and the *spleen* revealed leukaemic infiltrations with small accumulations of mature lymphocytes in the *portal tracts in the liver and in* the spleen, a rather diffuse infiltration with lymphocytes

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In retrospect the respiratory distress during the last days of life in both of our cases *taken together* with the *roentgenological* lung picture

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ACUTE HYPERTENSIVE VASCULAR DISEASE

1. *Relation between Blood Pressure Changes and Vascular Lesions in Different Forms of Acute Hypertension*

By

JØRN GIESE

Received 29.xi.64

Acute renal ischaemia in rats has been shown to elicit a syndrome characterized by a leakage of plasma from the intravascular compartment into the tissues and serous cavities together with a deposition of serum proteins in the media of small arteries and arterioles (*Giese* 1962). Further studies have shown that essentially the same syndrome can be elicited by the administration of rat kidney extracts, purified hog renin or synthetic angiotensin to nephrectomized rats (*Giese* 1963).

This paper reports studies aiming at a further elucidation of the mechanisms involved in the causation of this type of acute vascular damage and plasma leakage. Firstly, blood pressure recordings have been performed in rats exposed to the various experimental conditions mentioned above. Secondly, the effect of quinine on the blood pressure response induced by acute renal ischaemia was studied; administration of quinine has been shown to suppress the formation of serous effusions and tissue oedema in this experimental situation (*Giese* 1962). Thirdly, acute hypertension was induced by means of pressor agents other than renin or angiotensin; nephrectomized rats were used in order to exclude any participation of the renal renin system. The blood pressure responses and vascular lesions elicited in this way were studied for comparison with the experiments involving the renin angiotensin system.

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I wish to thank LEO Pharmaceuticals for supplies of heparin and Burroughs Wellcome & Co. (represented by Holger Andreasen, Copenhagen) for supplies of methoxamine.

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combined with antibiotics and cytostatics Clinically and morphologically the leukaemias were in a state of remission at death

At autopsy bilateral, massive Pneumocystis pneumonia was found to be the immediate cause of death in both cases The possibility that the combination of leukaemia and Pneumocystis pneumonia were not fortuitous is discussed *It is suggested that the combination of leukaemia and* intense treatment with corticosteroids and cytostatics by lessening the resistance prepared the ground for a massive fatal Pneumocystis pneumonia

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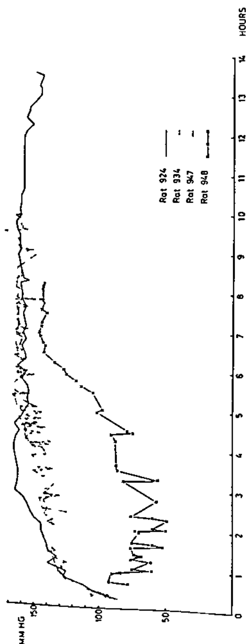


Fig 1
Records of blood pressure in four rats subjected to application of narrow silver clips on both renal arteries

MATERIAL AND METHODS

Animals Female albino rats each weighing approximately 200 grams were used

Blood pressure recordings were performed by means of a *Tybjærg Hansen* capacitance manometer, which was connected with a *Varian G-14 recorder* About 30 mm length of polyethylene tubing PE 10 (Clay – Adams) was connected to a 0 5 mm bore polythene cannula (Sterivac Allen & Hanburys Ltd) leading to the transducer The PE 10 tubing was inserted into the carotid artery and advanced towards the aorta Heparin saline (25 i u per ml) was used for filling the catheters and for flushing Electrical damping was mostly employed in order to obtain recordings of mean pressure Calibration pressures (0 and 180 mm Hg) were applied to the transducer approximately every 20 minutes

Induction of acute kidney ischaemia Clamps made of silver wire internal width 0 15 mm were applied on both renal arteries

Preparation of rat kidney extract Normal rat kidneys were homogenized in 4 ml of ice cold saline per gram of kidney tissue in a glass tissue grinder The homogenate was left for three days at 4° C for extraction After centrifugation dialysis against normal saline was performed in the cold The extract was stored at about —18° C and recentrifuged after thawing before use The final dilution was such that either 4 or 10 ml would contain the extractable non dialyzable substances from 1 gram of kidney tissue

Angiotensin Hypertensin CIBA (val^5 angiotensin II amide) was dissolved in normal saline to a concentration of 50 micrograms per ml

Noradrenaline a solution containing 0 2 mg of noradrenaline bitartrate (corresponding to 0 1 mg of noradrenaline) per ml was used

Methoxamine (β hydroxy β (2 5 dimethoxyphenyl) isopropylamine) was used as a solution of methoxamine hydrochloride containing 20 mg per ml (Vasoxine Burroughs Wellcome & Co)

Quinine was used as a 10 per cent solution of quinine hydrochloride dissolved in a 10 per cent solution of urethane

an automatic infusion machine
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sia After performance of nephrectomy or clamping of the renal arteries a subcutaneous injection of diethylbarbituric acid (30 mg of the sodium salt (barbital sodium)) was given this drug is excreted primarily through the kidneys and a very prolonged action of this single dose was evident in rats subjected to nephrectomy or kidney ischaemia thus making prolonged recording of blood pressure manageable without restraint

Histological technique The tissues were fixed in Bouins fluid Periodic acid Schiff staining (PAS) was used as a routine In some cases Mallorys phosphotungstic acid haematoxylin stain for fibrin (PTAH) and Massons trichrome stain in *Goldner's (1938) modification* was used

General remarks The duration of the experiments was generally about 24 hours *except where otherwise stated*

In the blood pressure records reproduced in this paper zero time is taken as the time at which nephrectomy was completed or (Figs 1 and 4) clamping of both renal arteries *had been performed*

RESULTS

A Studies on the Blood Pressure During the Development of Acute Vascular Disease

1 Acute Renal Ischaemia Induced by Bilateral Application of Narrow Silver Clips on the Renal Arteries

This procedure was performed in four rats the blood pressure was recorded for 8 14 hours after the operation as shown in Fig 1 A pronounced rise of the blood pressure was observed in three experiments the pressure rose to 150 160 mm Hg during the first four hours

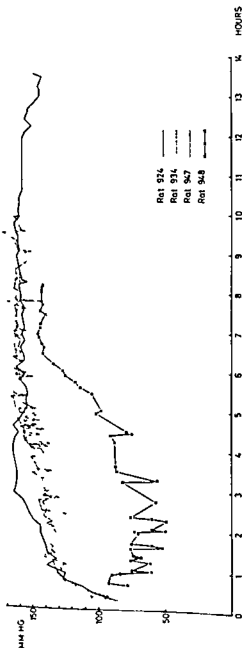


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Quinine was used as a 10 per cent solution of quinine hydrochloride dissolved in a 10 per cent solution of urethane

Intravenous infusions were given by an automatic infusion machine

Anaesthesia The operations involved in the experiments (clamping of renal arteries, nephrectomy, insertion of catheters) were performed under ether anaesthesia After performance of nephrectomy or clamping of the renal arteries a *subcutaneous injection of* diethylbarbituric acid (30 mg of the sodium salt (barbital sodium)) was given, this drug is excreted primarily through the kidneys and a very prolonged action of this single dose was evident in rats subjected to nephrectomy or kidney ischaemia thus *making prolonged recording* of blood pressure manageable without restraint

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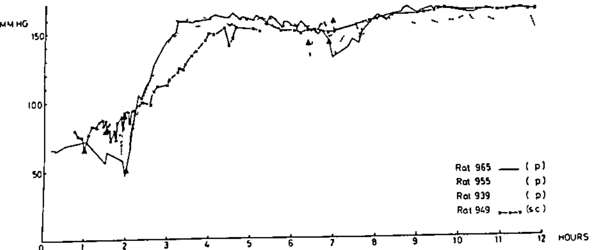


Fig. 2

Blood pressure changes in four nephrectomized rats after intraperitoneal (i p) or subcutaneous (s c) injections of rat kidney extract given at the times indicated by black triangles

and remained at this level for the rest of the period of observation In one rat (No 948) the pressure rise occurred much later, between the fifth and seventh hour, a level around 145 mm Hg being attained In two rats it was possible to resume the recording 22 to 25 hours after the operation One of these (No 934) showed a blood pressure slowly decreasing from 160 to 135 mm Hg over four hours, the other (No 924) had a blood pressure in the range 110 120 mm Hg

Autopsy was performed 21–27 hours after the operation A more or less complete infarction of both kidneys was observed signs of plasma leakage (oedema of mesentery and pancreas and small serous effusions) were found in all cases Microscopy showed the presence of PAS positive deposits in pancreatic or intestinal arterial vessels

2 *Administration of Rat Kidney Extract to Nephrectomized Rats*

In three experiments two intraperitoneal injections of rat kidney extract were given at 1½ 2 hours and 6½–7 hours after nephrectomy The volume injected was 5 ml containing extractable substances from 0 5 grams of normal rat kidney A rapid rise of the blood pressure was observed as demonstrated in Fig 2 Levels about 140 mm Hg after one hour and about 150–160 mm Hg after two hours were found the pressure staying at this level (150–160 mm Hg) for the remaining period of observation The blood pressure was recorded for 8 12 hours

In one experiment (rat 949) extractable substances from 1 gram of kidney tissue, contained in 4 ml of fluid were administered subcutaneously (multiple injection sites) The rise of blood pressure was somewhat slower, attaining finally approximately the same high level

Autopsy showed the previously described syndrome of plasma leakage and acute vascular disease

3 *Continuous Infusion of Synthetic Angiotensin into Nephrectomized Rats*

The dosage of angiotensin was approximately 1 7 micrograms per kg per min Three experiments were performed (Fig 3) recording trouble has interrupted one of the records The blood pressure rises at once at the start of the infusion to very high levels Later on the blood pressure stabilizes at different levels which are maintained for the period of observation The pressures recorded the next morning after about 21 hours of continuous infusion were about 105 mm Hg (rat 954), 125 mm Hg (rat 956) and 130 mm Hg (rat 1109)

Severe degrees of pancreatic and mesenteric oedema and typical vascular lesions with PAS positive deposits were found at autopsy

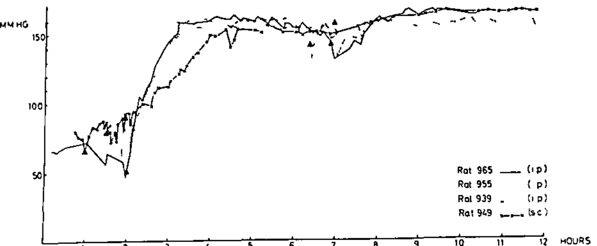


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B The Effect of Quinine on the Blood Pressure Response Following Bilateral Clamping of the Renal Arteries

Since it has been shown in an earlier paper (*Giese* 1962), that administration of quinine will suppress at least the macroscopic manifestations of plasma leakage after the induction of bilateral kidney ischaemia it seemed indicated to study the effect of quinine on the blood pressure response in this experimental situation

Three rats were subjected to bilateral application of 0 15 mm silver clips on the renal arteries quinine hydrochloride was administered in the dosage employed in the earlier experiments (two subcutaneous injections were given at one and six hours after the operation, respectively dosage 125 mg per kilogram of body weight given in each of the injections)

The recordings are depicted in Fig 4 they should be compared with the recordings in Fig 1 It is clearly seen that the blood pressure level is rather low in rats 1028 and 1029 not exceeding 120–125 mm Hg during ten hours of recording Rat 1005 shows a higher pressure level although never exceeding 140 mm Hg These results thus demonstrate that quinine in the dosage employed partly or totally prevents the development of high blood pressure after the induction of bilateral kidney ischaemia

Autopsy after about 24 hours showed no effusions or tissue oedema Microscopy showed no vascular lesions in rat 1028 and a few lesions in rats 1029 and 1005

C A Study on Morphological Changes in the Vascular System of the Rat Occurring After Induction of Acute Hypertension by means of Sympathomimetic Agents

The three types of acute hypertension studied in part A of this paper are all linked to the renin angiotensin system It now seemed of interest to study the effects of acute hypertension induced by means of other pressor agents in order to exclude any participation of the renal renin system the experiments were performed in nephrectomized rats Unfortunately the sustained hypertension observed in the experiments involving renin or angiotensin could not be duplicated by the administration of noradrenaline or methoxamine in the dosages employed Still some information was gained from these experiments

1 Infusions of Noradrenaline (about 3 4 Micrograms per kg per min)

In two experiments the infusion was given over approximately ten hours and the blood pressure was recorded during this period In a further five rats the infusion was continued for about 22 hours in three of these the blood pressure was recorded during the first five to

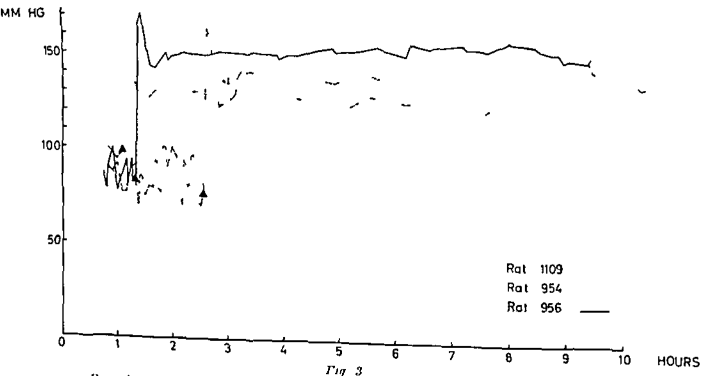


Fig 3

Rec r ls f 1 loo l pressure in three nephrectomize l rats receiving a continuous in fusi n of synthetic angiotensin (ab ut 1 7 micrograms per kg per min) Black triangles signal the start f inf isi n

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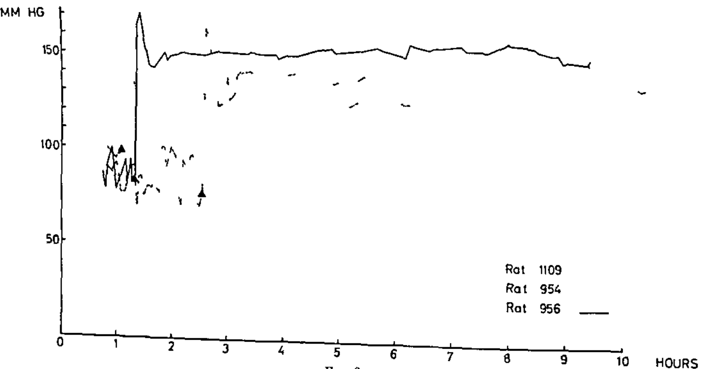


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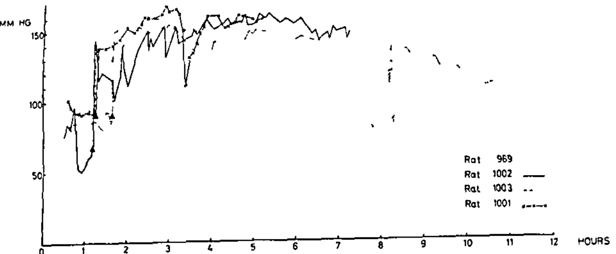


Fig 5

Records of blood pressure in four nephrectomized rats receiving noradrenaline (about 3 4 micrograms per kg per min) as a continuous infusion Black triangles signal the start of infusion

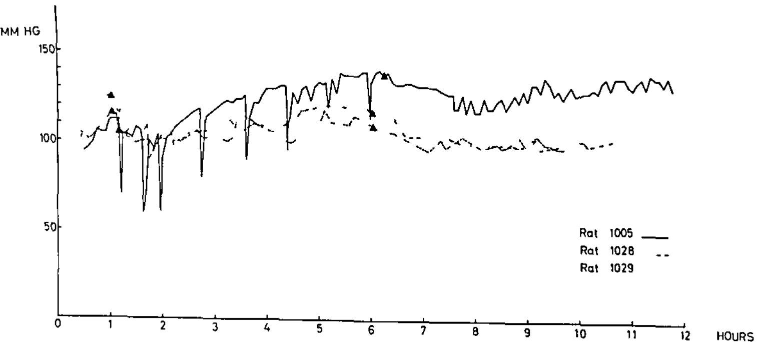


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Records of blood pressure in three rats subjected to bilateral clamping of the renal arteries and injected with quinine hydrochloride (125 mg per kg s c) at the times indicated by black triangles

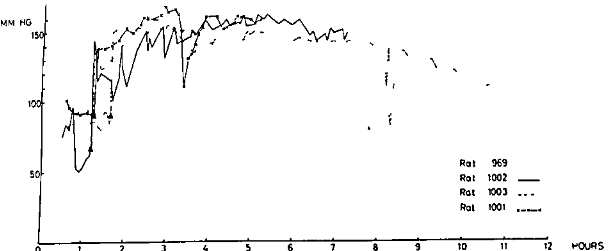


Fig 5

Records of blood pressure in four nephrectomized rats receiving noradrenaline (about 3.4 micrograms per kg per min.) as a continuous infusion. Black triangles signal the start of infusion



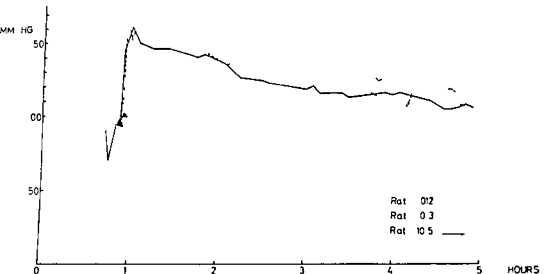
Fig 6

Rat 1001 Nephrectomy—infusion of noradrenaline Mesenteric artery showing fibrin-like deposits in the media Phosphotungstic acid —haematoxylin (× 720)

seven hours and again for a short period the next morning Autopsy was performed shortly after terminating the infusion

Recordings from four experiments are given in Fig 5 The blood pressure rises immediately at the start of the infusion, but the subsequent course is variable In some cases the pressure is fairly high for several hours (rats 969 – 1001 – 1002), in others the pressure declines (rat 1003) The pressure recorded after about 22 hours of infusion in three rats was in the range 80–100 mm Hg At the termination of the infusion a steep fall in pressure occurred

None of the rats showed the autopsy findings characteristic of renin- or angiotensin-induced vascular disease with plasma leakage, *i e* a generalized succulence of the abdominal organs, but slight degrees of localized mesenteric or pancreatic oedema were encountered in some of the rats In five of the rats no or only few vascular lesions were found, but two rats (No 969 and 1001, infused for 10 and 22 hours respectively) showed a fair number of PAS-positive deposits in arteries localized to the mesenteric border of the small intestine Deposits showing fibrin-like staining (dark blue) with Mallorys phosphotungstic acid—haema-



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Recor ls of 11 l ı re re in tl ree neı lrect mizc l rats receiving methoxaml e (115 140 micrograms per kg ı er min) as a continu ıs inf ısl n Black tr angles signal tl e start of ınfusi n

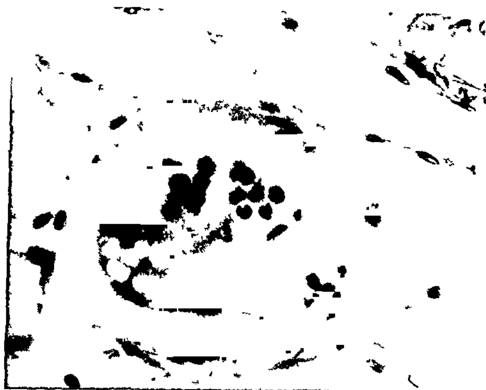


Fig 8
Rat 1012 Nephrectomy—*infusion of methoxamine Mesenteric artery showing* necrotic PAS positive media PAS stain (× 720)

toxylin procedure were demonstrable in a number of the damaged arteries (Fig 6) Small (rat 969) or quite substantial (rat 1001) fuchsinophilic deposits were found in some of the damaged arteries using *Goldner's* modification of Masson's trichome stain Some of the vessels showing deposits were necrotic, being devoid of nuclei in the PAS positive parts of the vessel walls

2 *Infusions of Methoxamine (115 140 Micrograms per kg per min)*

The dose level selected for these experiments proved to be rather toxic to the rats and 24 hour infusions were difficult to perform Accordingly only short term infusions were performed lasting four to five hours Five rats were infused one unanaesthetized four under light barbital anaesthesia In three rats the blood pressure was recorded during the experiment (Fig 7) A very high pressure is obtained initially, but the pressure decreases more or less rapidly

The most interesting autopsy finding was the presence of a pronouced oedema of the mesentery in four of the rats two of these showed a moderate degree of pancreatic oedema In these four rats vascular lesions were found in arteries located at the mesenteric border of the small intestine PAS positive deposits were found in the media

many of these small arteries were severely damaged, showing a homogeneous PAS-positive media with a few distorted nuclear remnants (Fig 8) No deposits could be demonstrated with the PTAH-stain or the trichrome-method The damaged vessels appeared much dilated in the sections

DISCUSSION

In the previous papers (*Giese* 1962 and 1963) forming the background of the present report, the development of plasma leakage and plasma protein deposits in the arterial walls was tentatively considered as a phenomenon indicating a general increase of endothelial permeability, occurring under circumstances combining the presence of renal insufficiency and the introduction of large amounts of renin into the circulation *Asscher & Anson* (1963) likewise considered the manifestations encountered after administration of rat kidney extract to nephrectomized rats to be caused by a permeability factor contained in the extract

The present experiments were performed in order to obtain more information concerning the relative importance of pressor effects and possible permeability-increasing effects of renin and angiotensin

The blood pressure recordings described in the first part of the paper show, that the application of narrow silver clips on both renal arteries is followed by the rapid development of acute hypertension The mean pressure rises to around 145–160 mm Hg and remains at this level for many hours Approximately the same level of sustained high blood pressure is attained when renin-containing rat kidney extract is administered to nephrectomized rats Continuous infusion of angiotensin into nephrectomized rats likewise induces a sustained elevation of blood pressure, although sometimes at a somewhat lower level In the few cases, in which the pressure recording could be resumed the next morning, the pressures were varying, sometimes still elevated, sometimes not This was not unexpected since the leakage of plasma from the vascular system must tend to lower the pressure All of the experimental animals showed autopsy findings indicating plasma leakage such as oedema of the mesentery and pancreas, and vascular lesions with PAS-positive deposits were found in the walls of pancreatic and mesenteric small arteries

It was known from previous studies (*Giese* 1962) that administration of quinine to rats exposed to acute bilateral kidney ischaemia would suppress the macroscopic manifestations of plasma leakage (tissue oedema and serous effusions) to a high degree These experiments were originally inspired by reports on the effectiveness of quinine as an "anti permeability agent" in experimental conditions involving increased capillary permeability (*Carone & Spector* 1960, *Spector & Willoughby* 1960) The blood pressure recordings under quinine treatment show, that a more or less pronounced hypotensive effect of

quinine in the dosage employed is quite evident This effect can be due to a myocardial or a direct vasodilator action of quinine Although these experiments do not exclude, that an "anti-permeability effect" of quinine can be of importance, they indicate that the suppression of pathologic manifestations may be due to the effect of quinine on the blood pressure

The effects of long-term infusions of *noradrenaline* have been studied in rabbits by *Blackel, Pickering & Wilson (1950)* and *Pugh, Pickering & Blackel* (1952) The initial hypertension was not maintained, and no vascular lesions were found In the present studies significant mesenteric arterial lesions were found in only two out of seven nephrectomized rats infused for 10–22 hours, but the lesions encountered show histological characteristics resembling renin- or angiotensin-induced vascular damage, including the presence of PAS-positive and fibrin-like deposits in the media

The induction of arterial necrosis by *methoxamine* was first described by *Herbertson & Kellaway* (1960), they described the frequent occurrence of pancreatic oedema and mentioned that fibrin-like material is found only occasionally in very small amounts in the necrotic arteries The present studies are in accordance with the original description, it is shown that the arterial lesions can develop in nephrectomized rats and the presence of—often severe—mesenteric oedema is noted It seems noteworthy, that oedema of the pancreas or mesentery has been found in several other experimental situations, all involving the presence of high blood pressure in rats with hypertensive encephalopathy (*Byrom* 1954), in rats subjected to bilateral renal ischaemia with infarction (*Giese* 1962) and in nephrectomized rats treated with rat kidney extract, *hog renin or synthetic angiotensin* (*Giese* 1963) The arterial lesions caused by methoxamine differ somewhat from the lesions induced by renin or angiotensin, no fibrin-like deposits can be identified by the *phosphotungstic acid – haematoxylin stain In evaluating the vascular* lesions it must be remembered, that the blood pressure decreases rather *rapidly in this type of acute hypertension*

The experiments reported in this paper cannot offer a final answer to the question, whether the renin-angiotensin system—under the experimental conditions described—induces an exudation of plasma into the serous cavities, *the abdominal tissues and the arterial* walls by way of the pressor effect or by way of a (hypothetical) permeability-increasing effect on the vascular endothelium But considered together these experiments *seem to support the first possibility A pronounced* and long maintained hypertension is the common finding in the three experimental situations studied in part A of this paper The effects of quinine on the *pathological manifestations might be explained by the* effect on the blood pressure The very acute occurrence of severe mesenteric or pancreatic oedema in methoxamine induced hypertension under circumstances where any *participation of the renal renin system has*

been excluded, would point to the prime importance of high intravascular pressure in the genesis of this manifestation of plasma leakage Likewise, the occasional finding of "fibrinoid" deposits in arterial walls after infusion of noradrenaline into nephrectomized rats tends to incriminate high blood pressure as the main responsible factor

SUMMARY

1 Blood pressure recordings for seven to twelve hours after the induction of severe renal ischaemia, after the administration of rat kidney extract to nephrectomized rats or during the continuous infusion of synthetic angiotensin into nephrectomized rats have demonstrated the occurrence of severe and sustained hypertension in all of the three experimental situations Acute vascular disease develops rapidly under these circumstances together with other signs of leakage of plasma from the intravascular compartment

2 Administration of quinine, which suppresses manifestations of plasma leakage after induction of bilateral kidney ischaemia, decreases the level of blood pressure in this experimental condition

3 Continuous infusion of noradrenaline into nephrectomized rats induces an acute hypertension of a varying stability Arterial necrosis with PAS positive and fibrin like deposits in the media was found in two out of seven rats after 10 to 22 hours of infusion

4 Infusion of methoxamine into nephrectomized rats induces a high blood pressure initially, but the pressure declines rather rapidly The severe vascular lesions encountered are described, signs of plasma leakage are frequently present

5 The experiments point to the prime importance of high intravascular pressure in the genesis of vascular lesions and plasma leakage

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3 *Byrom F B* The pathogenesis of hypertensive encephalopathy and its relation to the mal [illegible] dence from the hypert [illegible]

4 *Carone F A* [illegible] proteinuria in the ra [illegible] ermeability J Path [illegible] 80 [illegible] 1960

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quinine in the dosage employed is quite evident This effect can be due to a myocardial or a direct vasodilator action of quinine Although these experiments do not exclude, that an "anti-permeability effect" of quinine can be of importance, they indicate that the suppression of pathologic manifestations may be due to the effect of quinine on the blood pressure

The effects of long-term infusions of *noradrenaline* have been studied in rabbits by *Blackel, Pickering & Wilson* (1950) and *Pugh, Pickering & Blackel* (1952) The initial hypertension was not maintained, and no vascular lesions were found In the present studies significant mesenteric arterial lesions were found in only two out of seven nephrectomized rats infused for 10–22 hours, but the lesions encountered show histological characteristics resembling renin or angiotensin-induced vascular damage, including the presence of PAS positive and fibrin-like deposits in the media

The induction of arterial necrosis by *methoxamine* was first described by *Herbertson & Kellaway* (1960), they described the frequent occurrence of pancreatic oedema and mentioned that fibrin-like material is found only occasionally in very small amounts in the necrotic arteries The present studies are in accordance with the original description, it is shown that the arterial lesions can develop in nephrectomized rats and the presence of—often severe—mesenteric oedema is noted It seems noteworthy, that oedema of the pancreas or mesentery has been found in several other experimental situations, all involving the presence of high blood pressure in rats with hypertensive encephalopathy (*Byrom* 1954), in rats subjected to bilateral renal ischaemia with infarction (*Giese* 1962) and in nephrectomized rats treated with rat kidney extract, hog renin or synthetic angiotensin (*Giese* 1963) The arterial lesions caused by methoxamine differ somewhat from the lesions induced by renin or angiotensin, no fibrin-like deposits can be identified by the phosphotungstic acid – haematoxylin stain In evaluating the vascular lesions it must be remembered, that the blood pressure decreases rather rapidly in this type of acute hypertension

The experiments reported in this paper cannot offer a final answer to the question, whether the renin-angiotensin system—under the experimental conditions described—induces an exudation of plasma into the serous cavities, the abdominal tissues and the arterial walls by way of the pressor effect or by way of a (hypothetical) permeability-increasing effect on the vascular endothelium But considered together these experiments seem to support the first possibility A pronounced and long maintained hypertension is the common finding in the three experimental situations studied in part A of this paper The effects of quinine on the pathological manifestations might be explained by the effect on the blood pressure The very acute occurrence of severe mesenteric or pancreatic oedema in methoxamine-induced hypertension under circumstances where any participation of the renal renin-system has

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ACUTE HYPERTENSIVE VASCULAR DISEASE

2 *Studies on Vascular Reaction Patterns and Permeability Changes by means of Vital Microscopy and Colloidal Tracer Technique*

By

JØRN GIESE

Received 29 xi 64

In 1961 *Majno, Palade & Schoefl* published a study on chemical inflammation based on a method referred to as "vascular labelling", permitting the identification of leaking vessels by means of visible accumulations of foreign particles within their walls By the local injection of histamine or serotonin an increased exudation of plasma from the small vessels (in casu venules) is induced, if the plasma has been previously loaded with colloidal particles of carbon, these particles will be retained and accumulate within the wall of the leaking vessel, being too large to pass through the basement membrane Time is allowed for clearing of the blood stream by the reticulo endothelial system so that no carbon particles remain in the blood at the time of sacrifice The leaking vessels are thus outlined in black—a "vascular labelling" takes place

It was reasoned that since earlier studies (*Giese* 1962 and 1963) have shown an increased passage of fluid and plasma proteins through the vascular walls to be an essential step in the pathogenesis of acute hypertensive vascular disease, it ought to be possible to identify leaking arteries arterioles and smaller vessels at the same time and at an early stage of the disease by means of this technique This proved to be the case

Next this "vascular labelling" technique was used in combination with vital microscopy in order to study the process of labelling directly during its development *in vivo* This part of the study gave clear evidence concerning the relationship between the vascular reaction patterns observed after induction of acute hypertension and the occurrence of focal increases of vascular permeability

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This work was supported by grants from [illegible] nholdt W Jorck og Hustrus Fond [illegible] ' Seedorfs Legat P Carl Petersens [illegible]

[illegible] niversity Institute of Pathological [illegible] permission to use the ultramicrotome in his department I thank Dr *Poul Faarup* for cutting the thin sections of Vestopal imbedded tissue and especially for taking all photographs included in this paper

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MATERIAL AND METHODS

Animals Female albino rats each weighing about 200 grams were used

Blood pressure recordings and preparation of *rat kidney extracts* were performed as described in the preceding paper

Angiotensin Hypertensin CIBA (valyl angiotensin II-amide) was dissolved in physiological saline Generally a concentration of 50 micrograms per ml was used, in a few experiments (see Results part B, section 4) concentrations of 3 9 or 18 micrograms per ml were used

Noradrenaline a solution containing either 0 2 or 2 mg of noradrenaline bitartrate corresponding to 0 1 or 1 mg of noradrenaline per ml was used

Methoxamine was used as a solution of methoxamine hydrochloride containing 20 mg per ml ('Vasoxine', Burroughs Wellcome & Co)

Hydralazine Apresolin CIBA was used as a solution containing 20 mg per ml

Dihydralazine Nepresol CIBA was used as a solution containing 1 mg per ml

Colloidal carbon Carbon black especially prepared for experimental use (Guenther Wagner Pelikan Werke Hannover Germany, batch C 11/1431 a) This preparation is the same as used by *Majno Palade & Schoefl* (1961), according to these authors it contains about 100 mg/cc of carbon with an indicated average particle size of 200 A, it is stabilized with 4 5 per cent fish glue and contains 1 3 per cent phenol as a preserving agent

Intravenous infusions were given by an automatic infusion machine

Anaesthesia The operations involved in the experiments (nephrectomy insertion of catheters insertion of abdominal windows) were performed under ether anaesthesia After completion of nephrectomy or (when intact rats were used) at the beginning of the operation a subcutaneous injection of diethylbarbituric acid (30 or 40 mg of the sodium salt (barbital sodium)) was given the higher dose being used in the experiments involving vital microscopy In these experiments additional doses of barbital sodium (about 10 mg) were given intravenously when required at later stages of the experiment

Vital microscopy A very simple abdominal window was constructed based on parts of the more elaborate equipment described by *Palmer & Wilkins* (1960) A ring of plexiglas (external diameter 40 mm internal diameter 28 mm height 9 mm) was cemented to a circular plate of the same material (diameter 40 mm thickness 2 mm) The ring had an excavation around the whole circumference and was fitted with a plexiglas rod which could be fixed in a laboratory stand As described by *Palmer & Wilkins* the insertion of such a window is very simple, the abdomen is opened in the midline and the ring is inserted so that the edges of the abdominal muscles fit into the excavation like the tyre on the rim of a wheel The muscular edges are firmly fixed in this position by a thread encircling the excavation of the ring Incident light from a microscopy lamp was used observations were carried ou Electronic flash was used for photography in was used for fixation Clearing of tissues fo stereomicroscope was performed by means of methyl benzoate and oil of anise after dehydration in alcohols

In order to preserve the in vivo state of constrictions and dilatations in intestinal arteries for demonstration in a fixed preparation it was tried to perform instantaneous freezing of intestinal loops by decanting a freezing mixture (acetone/solid carbon dioxide) into the abdominal cavity of a deeply anaesthetized rat receiving a continuous infusion of angiotensin The frozen loops were removed and placed in absolute alcohol at 18° C for about one week to permit freeze substitution afterwards they were cleared by the usual method

For the preparation of thin sections tissue was embedded in Vestopal W after fixation in formalin and dehydration in acetone 1 micron sections were cut on a LKB ultramicrotome and affixed to glass slides coated with albumin glycerol Staining was performed with a 2 per cent aqueous toluidine blue solution

General remarks In all experiments polyethylene catheters were placed in the carotid artery (for blood pressure recording) in the jugular vein (for intravenous infusion of pressor agents) and *in the femoral vein (for administration of colloidal* carbon)

I wish to thank CIBA Pharmaceuticals Copenhagen for supplies of Apresolin and Nepresol

RESULTS

A Demonstration of Increased Vascular Permeability During Acute Hypertension Induced by Synthetic Angiotensin

Infusion of synthetic angiotensin into the nephrectomized rat has been shown to produce acute vascular disease with great regularity when suitable doses are employed (*Giese* 1963)

Six experiments were performed About one hour after nephrectomy an infusion of synthetic angiotensin (about 1 7 micrograms per kg per min) into the jugular vein was started Three hours later an injection of 0 2 ml of colloidal carbon was given through the femoral catheter without interrupting the angiotensin infusion which was continued for another hour after which the rat was decapitated under ether anaesthesia After centrifugation of the blood the serum did not contain visible carbon

At autopsy vascular labelling was macroscopically visible in five of the six rats Small arteries at the mesenteric border of the small intestine and at the intestinal surface were labelled in black in relation to these labelled arteries areas showing more diffuse deposits were found suggesting a labelling of smaller vessels When further studied by transillumination under a stereomicroscope (after fixation and clearing of the intestinal loops) the carbon deposits in the arteries were found to be localized in the media of the vessel arranged as parallel streaks in the vessel wall perpendicular to the long axis of the artery (Fig 1) often forming a ring like pattern encircling the artery (Fig 2) The deposits were found either as a more continuous investment for a variable distance or as focal cuffs around the artery A site of predilection was the mesenteric border of the small intestine but "cuff like deposits were often found in the course of the artery over the surface of the intestine In these preparations the labelled parts of the arteries always appe[illegible]

Thin secti[illegible] ers showed that in some pla[illegible] les were located correspond n[illegible] to the intercellular areas between the smooth muscle cells of the media More massive deposits of carbon were also found but it was difficult to make out the exact localization of these whether intra or extracellular (Fig 3) In some places carbon particles were found on either side of the internal elastic membrane the relationship of the luminally situated particles to the endothelial cytoplasm could not be made out

The areas showing diffuse deposits indicating abnormal leakiness of smaller vessels were predominantly located in close relation to labelled arteries [illegible]
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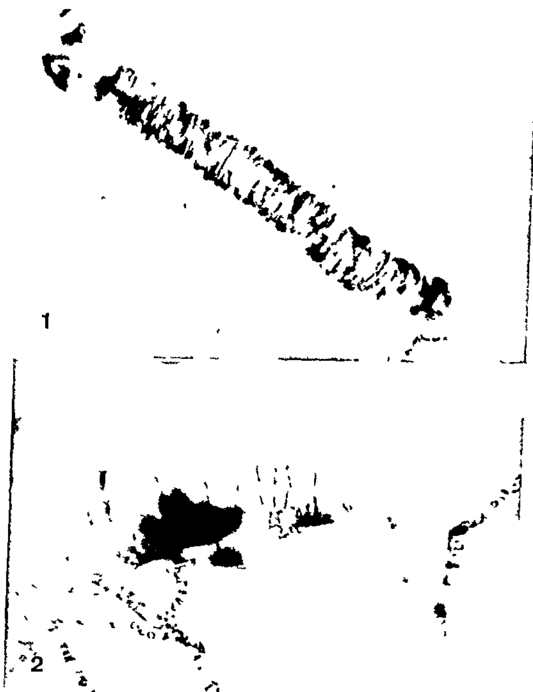
I wish to thank CIBA Pharmaceuticals Copenhagen for supplies of Apresolin and Nepresol



Figs 3 4

Fig 3 Rat 1033 Nephrectomy angiotensin infusion Formalin fixation embedding in Vestopal W 1 micron section Intestinal artery showing deposits of carbon in the media Some of the discrete deposits are located in the intercellular areas between the smooth muscle cells More massive deposits are also seen the localization of these is uncertain Toluidine blue stain (× 1800)

Fig 4 Rat 1033 Nephrectomy—angiotensin infusion Preparation as in Fig 3 Section from the intestinal wall showing a small vessel containing carbon in the wall Toluidine blue stain × 1800



Figs 1 2

Fig 1 Rat 1033 Nephrectomy—angiotensin infusion Formalin fixed and cleared preparation Mesenteric artery showing heavy deposits of carbon in the wall arranged in streaks perpendicular to the long axis of the vessel (× 184)

Fig 2 Rat 1033 Nephrectomy—angiotensin infusion Preparation as in Fig 1 Arteries at the mesenteric border of the small intestine demonstrating pronounced carbon deposits in ring like arrangements around the circumference of the vessels A labelled dilated branch leads to an area of heavy diffuse labelling (× 46)

was hard to make out in paraffin sections in a limited number of thin Vestopal sections occasional small vessels were observed showing a deposition of carbon in the wall (Fig 4)

Since the continuous measurement of the blood pressure necessitates a varying number of flushings with saline through the carotid catheter in order to keep it patent and since these injections of saline into the arterial system might possibly be of importance the experiment was repeated in five rats treated in exactly the same way except that the carotid artery was not cannulated and no blood pressure recording was performed Of these five rats four showed deposits of carbon in intestinal arteries and diffuse deposits in smaller vessels

The experiments were further repeated in five nephrectomized rats receiving an infusion of physiological saline instead of angiotensin The blood pressure was around 100 mm Hg in four of these rats no arterial deposits of carbon and only few areas showing deposits in minute vessels were found in two of the rats the endothelial surface of a few small veins was coated with carbon One rat had an unusually low blood pressure (around 60 70 mm Hg) and showed several areas of diffuse labelling together with coating of the endothelial surface of a few small veins with carbon these changes were confined to a small section (length about 6 cm) of the intestine No arterial deposits were found

Blood pressure recordings from two angiotensin experiments and one control experiment are shown in Fig 5

B Vital Microscopy of Intestinal Arteries During Acute Hypertension *Vascular Reaction Patterns and Permeability Changes*

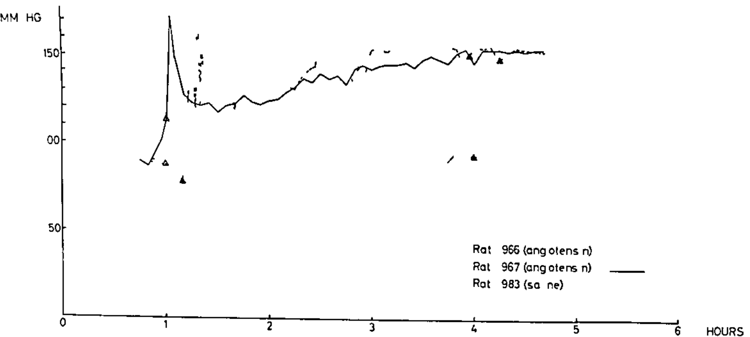
The use of an abdominal window offered excellent possibilities for microscopical observations during several hours Several intestinal arteries can be studied in the field of vision and no signs of damage to the intestines have been observed

A number of experimental situations were studied by the combination of vital microscopy and the vascular labelling technique

1 *Infusion of Angiotensin (about 1 7 Micrograms per kg per min) into Nephrectomized Rats*

This method for induction of acute hypertension was chosen for primary studies in order to obtain more information concerning the mechanisms involved in the development of the permeability changes described in part A of this paper

Fourteen experiments were performed generally the infusion of angiotensin was started one to two hours after nephrectomy when the catheters and the abdominal window had been inserted The blood pressure was measured continuously



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Recor ls of l lood pressure in two ang otens n experiments an l one control experi ment White triangles start f nf s on l lack triangles injecti n f colloidal car l on See text for furtl er explanat on

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Figs 6–7

Fig 6 Rat 1047 Nephrectomy—angiotensin infusion Intestinal loop prepared by immediate freezing—freeze substitution in alcohol—clearing The characteristic pattern of dilatations (one fusiform one located in a bifurcation) with intervening constricted parts in the course of the artery is seen (× 45)

Fig 7 Rat 1047 Nephrectomy—angiotensin infusion Another region of the preparation used in Fig 6 Several dilatations are seen including dilated parts of the mesenteric arterial arcade separated by constricted segments (× 45)

Figs 8–11

Fig 8 Rat 1070 Intact kidneys—angiotensin infusion Photograph taken *in vivo* through the stereomicroscope Two pronounced dilatations in the region of the artery close to the mesentery are separated by a short zone of constriction From the distal dilated segment a side branch goes off, showing dilatation tapering into a severely constricted part (× 60)

Fig 9 Rat 1078 Intact kidneys—angiotensin infusion *In vivo* photograph Intestinal artery showing moderately dilated region with several carbon deposits in the lower part of the picture The left branch is dilated and labelled in the first part thereafter constricted the right branch shows some labelled regions along the course (× 60)

Fig 10 Rat 1078 Intact kidneys—angiotensin infusion *In vivo* photograph Small artery showing labelled regions which are moderately dilated The intervening constricted parts had begun to relax at the time of photography (× 60)

Fig 11 Rat 1078 Intact kidneys—angiotensin infusion In vivo photograph Intestinal artery showing several carbon deposits The heavy deposit in the proximal dilated part of the side branch going off towards right is especially noteworthy, since the first deposit in this region was observed 50 seconds after the start of the angiotensin infusion carbon having been injected just before the start of the infusion (× 60)


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Fig 12

Rat 1047 Nephrectomy—angiotensin infusion Detail from Fig 6 A single streak like deposit of carbon particles in the wall of a dilated region in an intestinal artery (× 175)

In twelve of the experiments a very characteristic pattern of alternating constrictions and dilatations along the course of the intestinal arteries developed immediately at the start of the angiotensin-infusion, simultaneously with the pronounced rise of blood pressure (Figs 6 and 7) The proximal parts of the visible course of the large and small arteriae rectae, i e the region close to the mesenteric border of the intestine, were the seat of often pronounced dilatation sometimes two dilatations, separated by a short constricted zone, could be observed (Fig 8) Dilatations, separated by severely constricted segments, were seen in the course of the artery distally towards the antimesenteric border (Figs 6 and 7) Besides the dilatations observed in the main trunks such dilatations were rather often found in side branches, the proximal dilated part of the branch tapering into a pronounced constriction (Fig 8) Some of the visible arteries would show a more uniform constriction without dilatations, still others looked widely open,

often showing slight additional dilatations in several places In many cases a very large percentage of the visible arteries showed the pattern of focal spasm alternating with dilated segments when the observations were continued it was found that in some rats the constrictions and dilatations initially formed at the start of the angiotensin infusion would persist in the same location for several hours whereas in other rats the pattern would disappear in some or most of the initially affected vessels

Injections of colloidal carbon (0 1 or 0 2 ml) were given through the femoral vein catheter at different times during the run of the infusion often about 15 minutes after the start of the experiment sometimes 0 1 ml was given at later stages The injection imparted a black colouring to the blood stream incidentally allowing the observation that none of the severely constricted parts were completely closed up since the carbon spread freely over the whole of the visible vasculature After a short time the black colour became of decreasing intensity thus allowing carbon deposits in the vascular walls to be recognized The important finding repeated in all experiments where carbon deposits were found was that *deposits of carbon are never found in the spastic constricted parts of the arteries* but *predominantly in clearly dilated segments* (Figs 9 10 11 12) A definite site of predilection was the proximal dilated part of the intestinal arteries but very often dilatations in the course of the artery over the intestinal surface showed carbon deposits Some deposits were observed in apparently widely open arteries (without constricted segments) especially in slight dilatations on the otherwise even contour The deposits were seen as streaks or dots in the wall of the artery just as described for the fixed and cleared preparations in part A of this paper When the injection of carbon was repeated at later stages additional deposits were often formed either in already labelled segments or in not hitherto labelled dilatations

In two of the experiments very few arteries showed spasm and dilatations Only a single deposit was observed in a dilated segment

In some experiments diffuse labelling indicating increased permeability of minute vessels was observed just as seen in the fixed preparations At times these deposits were located in parts of the intestine where the arteries seemed relatively open without severe constriction

The degree of arterial and diffuse labelling was quantitatively much varying in the different experiments In many experiments a very large number of dilated segments were visible but by no means all of these became labelled

9 *Infusion of Angiotensin (about 1 7 Micrograms per kg per min) into Intact Rats*

The experiment was next repeated in four rats with intact kidneys In three of these infusion of the same high dose of angiotensin imme

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diately produced the same pattern of constrictions and dilatations as seen in the nephrectomized group, and injections of carbon gave rise to the formation of mural deposits exclusively in dilated segments

In two of these experiments diffuse labelling occurred, either close to dilated proximal segments of the arteries or related to arterial trunks without severe constrictions

The described phenomena are thus readily inducible in rats with intact kidneys

3 *Determination of Time Intervals Needed for the Formation of Mural Deposits of Carbon in the Intestinal Arteries*

In four experiments 0 1 ml of colloidal carbon was injected about one minute before the infusion of angiotensin (same dosage) was started A stop watch was started when the blood pressure rise occurred, and the *field of vision was closely searched, with this dose of carbon it is* quite possible to recognize mural deposits even when carbon particles are circulating in the blood stream

In one rat with intact kidneys the first definite deposit was observed after 50 seconds (Fig 11), the next after 90 seconds, after five minutes several deposits were visible in dilated parts In another experiment the first deposits were found after 150, 180 and 210 seconds

Two experiments were performed in nephrectomized rats The first three deposits were observed after 230, 275 and 330 seconds in one of these, the other rat showed very few deposits, the first one being recognized 420 seconds after the start of the angiotensin infusion

Hyperpermeability of dilated segments of the intestinal arteries can thus be recognized during the first few minutes after the induction of acute severe hypertension

4 *Infusion of Lower Doses of Angiotensin (0 1 0 3–0 6 Micrograms per kg per min) into Rats with Intact Kidneys*

Five experiments were performed At the two lower dosages only *moderate constrictions and dilatations were visible A single carbon* deposit was recognized in a moderately distended segment in one of these three experiments At the highest dosage one rat showed the previously described constriction-dilatation pattern with subsequent carbon labelling of dilated parts, the other rat showed a rather uniform vasoconstriction and no deposits were observed No clear relationship between dosage and pressure level was found

5 *Effects of Lowering the Blood Pressure during Infusion of Angiotensin into Nephrectomized Rats*

The previously described pattern of constrictions and dilatations was induced by infusion of *angiotensin (about 1 7 micrograms per kg per* min)

The *effects of stopping the infusion* was studied in three rats The blood pressure decreased gradually and a gradual disappearance of the constrictions and dilatations was observed, after 5–10–15 minutes the pattern was no more recognizable In two of these rats the infusion was then started again, the constrictions and dilatations reappeared in many of the arteries, apparently in the same localizations as before

The *effects of bleedings and retransfusions* were studied in three rats, with the angiotensin infusion running continuously the arterial pressure was lowered by bleeding of the rat by way of an additional catheter placed in the femoral artery. The dilated segments showed a progressive diminution of diameter and the constricted parts showed a further narrowing, but the pattern of alternating spasm and dilatation could be recognized in some arteries at least for a time after reaching normal or subnormal pressures, tending finally to disappear leaving the arteries as severely constricted strings With subsequent retransfusion the pattern could be reestablished in most of the arteries in two of the rats, in most places the constricted and dilated segments seemed to reappear at the same locations along the course of the arteries as before the bleeding procedure

Even with this heavy dosage of angiotensin, a steep pressure fall occurs in spite of continued infusion if 0 2 mg of *hydralazine* (Apresolin CIBA) is injected intravenously The pattern of constrictions and dilatations was observed in two rats during the pressure fall (from 140–150 mm Hg down to 50–60 mm Hg in six to eight minutes) induced in this way, the pattern disappeared within about five minutes

In order to study the permeability characteristics of the intestinal arteries in rats showing no elevation of the blood pressure in spite of continuous administration of angiotensin ten additional experiments were performed In the first five of these acute hypertension with the associated constriction/dilatation pattern was induced by means of angiotensin in the usual dosage, and the angiotensin infusion was kept running during the whole of the experiment After five m n [illegible]

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by 0 10 mg) was

mm Hg to about

[illegible] mm Hg within 10 minutes At this time an injection of colloidal carbon was given

After the injection of Nepresol the severely spastic segments could be observed to open up and many arteries regained their normal contour, but in some arteries the dilatations formed at the start of the experiment were still visible after normal blood pressure values had been obtained In one of the rats practically all arteries regained their normal appearance and no labelling took place In two rats one or two carbon deposits were observed, localized to persistent dilatations Finally, two rats showed a fair number of carbon deposits Some of these were localized in typical dilatations, separated by intervening, more narrow

diately produced the same pattern of constrictions and dilatations as seen in the nephrectomized group, and injections of carbon gave rise to the formation of mural deposits exclusively in dilated segments

In two of these experiments diffuse labelling occurred, either close to dilated proximal segments of the arteries or related to arterial trunks without severe constrictions

The described phenomena are thus readily inducible in rats with intact kidneys

3 *Determination of Time Intervals Needed for the Formation of Mural Deposits of Carbon in the Intestinal Arteries*

In four experiments 0 1 ml of colloidal carbon was injected about one minute before the infusion of angiotensin (same dosage) was started A stop watch was started when the blood pressure rise occurred, and the field of vision was closely searched, with this dose of carbon it is quite possible to recognize mural deposits even when carbon particles are circulating in the blood stream

In one rat with intact kidneys the first definite deposit was observed after 50 seconds (Fig 11), the next after 90 seconds, after five minutes several deposits were visible in dilated parts In another experiment the first deposits were found after 150, 180 and 210 seconds

Two experiments were performed in nephrectomized rats The first three deposits were observed after 230, 275 and 330 seconds in one of these, the other rat showed very few deposits, the first one being recognized 420 seconds after the start of the angiotensin infusion

Hyperpermeability of dilated segments of the intestinal arteries can thus be recognized during the first few minutes after the induction of acute severe hypertension

4 *Infusion of Lower Doses of Angiotensin (0 1 0 3 0 6 Micrograms per kg per min) into Rats with Intact Kidneys*

Five experiments were performed At the two lower dosages only moderate constrictions and dilatations were visible A single carbon deposit was recognized in a moderately distended segment in one of these three experiments At the highest dosage one rat showed the previously described constriction dilatation pattern with subsequent carbon labelling of dilated parts, the other rat showed a rather uniform vasoconstriction and no deposits were observed No clear relationship between dosage and pressure level was found

5 *Effects of Lowering the Blood Pressure during Infusion of Angiotensin into Nephrectomized Rats*

The previously described pattern of constrictions and dilatations was induced by infusion of angiotensin (about 1 7 micrograms per kg per min)

The *effects of stopping the infusion* was studied in three rats. The blood pressure decreased gradually and a gradual disappearance of the constrictions and dilatations was observed, after 5–10–15 minutes the pattern was no more recognizable. In two of these rats the infusion was then started again, the constrictions and dilatations reappeared in many of the arteries, apparently in the same localizations as before.

The *effects of bleedings and retransfusions* were studied in three rats: with the angiotensin infusion running continuously the arterial pressure was lowered by bleeding of the rat by way of an additional catheter placed in the femoral artery. The dilated segments showed a progressive diminution of diameter and the constricted parts showed a further narrowing, but the pattern of alternating spasm and dilatation could be recognized in some arteries at least for a time after reaching normal or subnormal pressures, tending finally to disappear leaving the arteries as severely constricted strings. With subsequent retransfusion the pattern could be reestablished in most of the arteries in two of the rats: in most places the constricted and dilated segments seemed to reappear at the same locations along the course of the arteries as before the bleeding procedure.

Even with this heavy dosage of angiotensin, a steep *pressure fall* occurs in spite of continued infusion if 0.2 mg of *hydralazine* (Apresolin CIBA) is injected intravenously. The pattern of constrictions and dilatations was observed in two rats during the pressure fall (from 140–150 mm Hg down to 50–60 mm Hg in six to eight minutes) induced in this way: the pattern disappeared within about five minutes.

In order to study the permeability characteristics of the intestinal arteries in rats showing *no elevation* of the blood pressure in spite of continuous administration of angiotensin ten additional experiments were performed. In the first five of these acute hypertension with the associated constriction/dilatation pattern was induced by means of angiotensin in the usual dosage and the angiotensin infusion was kept running during the whole of the experiment. After five minutes an intravenous injection of *dihydralazine* (Nepresol CIBA) in a dose of 0.35 mg (in one case 0.25 mg followed later by 0.10 mg) was given eliciting a gradual pressure fall from 170–140 mm Hg to about 100 mm Hg within 10 minutes. At this time an injection of colloidal carbon was given.

After the injection of Nepresol the severely spastic segments could be observed to open up and many arteries regained their normal contour but in some arteries the dilatations formed at the start of the experiment were still visible after normal blood pressure values had been obtained. In one of the rats practically all arteries rega[illegible]

[illegible] deposits. Some of these were localized in typical dilatations separated by intervening more narrow

segments, other deposits were found to occur in apparently widely open arteries, especially in very slight dilatations on these vessels During the period in which the carbon particles were circulating, the blood pressure was in the range 70–115 mm Hg It would thus seem, that the persistence of an abnormal pattern in the vasculature may allow a limited number of mural deposits to be formed even with the systemic arterial pressure inside the normal range

In five experiments 0 35 mg of Nepresol was given 10 minutes before the start of the angiotensin infusion, the pressure was lowered to around 40–50 mm Hg When the angiotensin infusion was started the pressure rose sharply to 140–150 mm Hg, but only for a few minutes, and the pattern of constrictions and dilatations disappeared quickly in four of the rats The angiotensin infusion was kept running, but the pressure was kept in the range 60–125 mm Hg by the effect of dihydralazine Colloidal carbon was given after 15 minutes of angiotensin infusion, but no deposits were found in the arteries observed In one rat, however, although the blood pressure was well controlled (not exceeding 90 mm Hg), the constriction/dilatation-pattern did not disappear completely Two carbon deposits were observed in an intestinal artery, lying in an open segment, the branches of which were constricted further distally

6 *Administration of Rat Kidney Extract to Nephrectomized Rats*

Rat kidney extract was administered subcutaneously to one, intravenously to another nephrectomized rat In both moderate constrictions and dilatations were found to develop in some arteries, with subcutaneous administration these changes developed very slowly and were not pronounced until six hours after the injection of kidney extract, although the blood pressure had attained a level of 150 mm Hg after 1½ hours and 160 170 mm Hg after 3½ hours After injection of carbon labelling occured, again only in dilated segments

7 *Administration of Noradrenaline to Nephrectomized Rats*

Two experiments were performed using the same dosage as in the experiments reported in the preceding paper (about 3 4 micrograms per kg per min) The vasoconstriction was more uniform than with infusion of angiotensin, but a few moderately distended segments were observed in the proximal parts of the intestinal arteries labelling with carbon was observed only in such dilated segments

Three experiments were performed with a dosage of 34 micrograms per kg per min, the blood pressure decreased rapidly after an initial pronounced rise In one rat constrictions and dilatations were visible initially, but after a short time the arteries were rather uniformly constricted Two rats showed a pronounced constriction/dilatation pattern in some of the visible arteries, much like that seen with angiotensin

Carbon deposits were found in pronounced dilatations of the proximal parts of the arteries and in dilated segments further distally, also diffuse deposits in minute vessels were found

8 *Infusion of Methoxamine into Nephrectomized Rats*

As a further parallel to the studies reported in the preceding paper, methoxamine infusion (about 125 micrograms per kg per min) was performed in three nephrectomized rats Varying types of reaction were observed in the intestinal arteries, which could show a uniform constriction or a pronounced dilatation in the proximal region with severe constriction in the more distal parts or the pattern of alternating constricted and dilated segments along the course of the artery

Carbon deposits were predominantly formed in the proximal distended segments and occasionally in more distally located dilatations No deposits were seen in the constricted segments Diffuse deposits were found in two of the experiments as a sign of increased permeability in smaller vessels

9 *Control Experiments Infusion of Saline into Nephrectomized Rats*

Two experiments were performed in nephrectomized rats receiving intravenous saline infusion in the same volume (about 0 4 ml per hour) as used in the angiotensin experiments No spasm or pronounced dilatation was observed during four to five hours of observation, in one of the rats a single artery showed four minor dilatations in the beginning of the experiment, but they disappeared after a short time Carbon injections after three or four hours were not followed by any formation of deposits

DISCUSSION

The phenomenon referred to as vascular labelling' [illegible]

[illegible] process indicates a local increase of permeability The carbon particles are used as a *colloidal* tracer substance indicating the pathways followed by *plasma* escaping from the intravascular compartment and the "vascular labelling phenomenon constitutes a parallel to the previously *described* occurrence of deposits of fluorochrome tagged serum proteins in arterial walls during repeated episodes of acute hypertension (*Giese 1961*)

The most important fact *derived from* the combined application of vital microscopy and the vascular labelling technique to the study of acute hypertension is the very *consistent* observation, that the deposition of carbon in the arterial wall takes place preferentially *in dilated*

segments of the small arteries, carbon deposits were never observed in severely constricted regions The sites of predilection for the formation of mural deposits were the distended proximal segments of the intestinal arteries and *pronounced dilatations further distally*, at times deposits were observed in uniformly open arteries, often in additional small dilatations along the course of the vessel

When trying to understand, why the increased permeability of the arterial walls is manifested only in dilated or at least widely open segments of the intestinal arteries, whereas segments showing constriction are never affected, some basic difficulties are encountered In the first *place, no precise explanation is available concerning the question why* the arteries react in this peculiar way, showing constrictions in some places, dilatations in other places, when the vascular tone and the intraluminal pressure is increased by the administration of pressor agents Secondly, although the systemic arterial pressure has been recorded, the intraluminal pressure in a given segment showing labelling is unknown in the absence of direct measurement On the other hand it is quite clear, that the tangential tension in the wall of a dilated segment can be several times larger than the tension in the wall of an immediately adjacent constricted segment, since this tension is determined by the product of the intraluminal pressure and the radius (*Wolf* 1952)

The most reasonable assumption to be derived from the experimental findings would seem to be, that the essential factor is a distension of the structures of the arterial wall The anatomical structures so influenced have not been identified in this study, but it would be reasonable to suggest changes in the intercellular endothelial junctions, the internal elastic lamina or/and the subendothelial basement membranes

This concept, putting the induction of increased permeability of the arterial wall down to physical forces, would make the assumption (*Giese* 1962 and 1963), that the renin-angiotensin system might have a general permeability-increasing effect on vascular endothelium, rather untenable Also, the experiments have shown, that focal increases of vascular permeability may be induced by other pressor agents

When evaluating the experiments showing, that a limited number of carbon deposits may be formed in still dilated or at least widely open arteries during the infusion of angiotensin even at a time, when the blood pressure has been lowered by pharmacological means, it must be recognized that the haemodynamic situation created in a circulatory system under the joint influence of a pressor and a vasodilator agent is rather complex, and again direct measurement of the pressure locally in a given segment showing deposits would be necessary for a more complete analysis But the decisive point seems to be, that an abnormal pattern is maintained in the vasculature by angiotensin even after lowering of the systemic arterial pressure

Carbon deposits in the wall of *small arteries have been recognized*

within the first few minutes after the induction of acute hypertension with angiotensin This astonishing rapidity in the development of hyperpermeable parts of the arterial system points strongly to the assumption that a deposition of plasma constituents in the vascular wall is a primary occurrence in the chain of events leading to acute hypertensive vascular damage The very short time intervals needed for the development of mural deposits make it highly unlikely that the development of increased permeability should be regarded as a phenomenon dependent upon prior degeneration of the smooth muscle cells of the media It seems much more likely that the focal hyperpermeability develops at once when the necessary physical conditions come into presence

Besides the carbon deposits in the arterial walls diffuse deposits of carbon indicating foci of increased permeability in smaller vessels have been observed during vital microscopy and in fixed and cleared preparations This finding is in accordance with the frequent presence of mesenteric and intestinal oedema in rats exposed to angiotensin (and methoxamine) These diffuse deposits are very often located in close relation to dilated labelled arteries especially at the mesenteric border and often such dilated arteries seem to lead directly to the diffuse deposits (Fig 2) No precise definition of the type of minute vessels involved in the formation of these deposits has been obtained and the observations do not permit final conclusions concerning their pathogenesis A reasonable hypothesis for further study would be that the increased permeability of these small vessels is due to an *elevation of* intraluminal pressure dilatation under high pressure of the artery supplying the area in question might allow the high intraarterial pressure to be transmitted distally thus allowing a pressure rise with dilatation and increased permeability of these small vessels This concept would also fit well with the observations during vital microscopy

The studies reported here are only concerned with observations during very acute hypertension Therefore it is of the greatest interest that *Byrom* (1954 1958,59) has described the occurrence of a pattern of alternating severe constrictions and dilatations in the intestinal arteries of rats suffering from severe renal hypertension with or without hypertensive encephalopathy His pictures of the intestinal vascular pattern are completely like the vital microscopic picture observed after induction of acute hypertension with angiotensin The pattern of focal spasms and focal dilatations has further been observed in the cerebral arteries *Byrom* 1954) during hypertensive encephalopathy and a similar pattern was observed in the *retinal arteries* of hypertensive rats by the same author (*Byrom* 1963)

Byrom's concept that the vascular lesions in human and experimental malignant hypertension are caused by repeated vascular crises involving a state of severe spasm seems very well supported by his elegant investigations In all of the *experimental* situations studied by

segments of the small arteries, carbon deposits were never observed in severely constricted regions The sites of predilection for the formation of mural deposits were the distended proximal segments of the intestinal arteries and pronounced dilatations further distally, at times deposits were observed in uniformly open arteries, often in additional small dilatations along the course of the vessel

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Carbon deposits in the wall of small arteries have been recognized

7 *Majno G Palade G E & Schoefl G I* Studies on inflammation II The site of action of histamine and serotonin along the vascular tree a topographic study J biophys biochem Cytol *11* 607 1961
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Byrom the occurrence of focal spasm has been frequently accompanied by the development of focal dilatations of the arteries, the same was regularly the case in the experiments reported in the present paper If applicable to less hyperacute forms of hypertension, the investigations reported here would show, that the spastic arterial segments themselves are protected against plasmatic vasculosis, which affects only the distended parts of the small arterial vessels

SUMMARY

1 Demonstration of increased permeability in small arteries and minute vessels during acute hypertension induced by synthetic angiotensin was possible using the "vascular labelling" technique of *Majno, Palade & Schoefl* This method permits the identification of leaking vessels by means of visible accumulations of carbon particles within the vessel wall

2 The vascular labelling method was combined with vital microscopy, using an abdominal window The vascular reactions observed during acute hypertension induced by angiotensin, noradrenaline and methoxamine are described Infusion of angiotensin very often gives rise to a characteristic pattern of alternating constrictions and dilatations A deposition of carbon particles from the blood stream occurs only in the walls of dilated arterial segments, deposits are never found in severely constricted parts of the arteries The development of hyperpermeable regions of intestinal arteries has been observed as early as during the first few minutes after induction of acute hypertension

3 During continued infusion of angiotensin a persistence of an abnormal vascular pattern even after lowering the arterial pressure to approximately normal values has been observed in some experiments In such cases a limited number of deposits can be formed in the persistent dilatations

4 It is concluded, that the essential factor allowing a passage of colloidal particles (and, by inference, plasma proteins) into the arterial wall is a distension of the vessel wall

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6 *Giese* [illegible] caused by acute renal ischaemia Acta [illegible] 7 1963

droxytryptophan in large amounts and of inducing the carcinoid syndrome (*Sandler et al* 1961), but the agentaffin reaction is nonetheless often negative in bronchial carcinoids *Weiss & Ingram* (1961), for example, observed a positive argentaffin reaction in but one of 5 bronchial carcinoids

Clearly, therefore, some carcinoids for obscure reasons exhibit a negative argentaffin reaction In most papers on negative argentaffin reactions it is not mentioned whether other histochemical procedures for demonstrating enterochromaffin were attempted Hence, in order to seek to elucidate the significance of negative argentaffin reactions in carcinoids, it was deemed worth while to compare the results of various histochemical reactions in a fairly large series of carcinoids The methods were checked by applying them to enterochromaffin cells in normal human appendix

MATERIAL AND METHODS

The investigation was performed on a series of 27 carcinoids from the tumour register of the Pathological Laboratories Gothenburg *The series included all the* carnoids diagnosed by operative biopsy over the years *1954 through 1963* after elimination of any cases in which the diagnosis was [illegible]
grounds Carcinoids found at autopsy wer[illegible]
chromaffin substance is known to disapp[illegible]
The material was available *in the form of*
ently fixed in formalin Sections 4 μ thic[illegible] [illegible] *weak reactions*

metheneamine-
incubation at
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placing sect[illegible]

ing impregnation of the
bservations and demon-
precisely controlled in
reaction Any carcinoid showing a
negative reaction after 140 minutes' *incubation* was also examined after 160 minutes' incubation when the connective tissue *stroma of* [illegible]

with
ined
90

[illegible] several stable diazo compounds tested the best [illegible]

[illegible] colour the enterochromaffin granules and carcinoid granules orange to red and the background yellow The intensity of the *background* colour increases with lengthening *incubation* periods impairing the demonstrability of reactive material With the incubation period adopted here *the background* takes on a pale

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ENTEROCHROMAFFIN REACTIONS IN INTESTINAL CARCINOID TUMOURS

By

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Received 21 ii 64

Since both enterochromaffin cells and carcinoid tumour cells after formalin fixation exhibit granules with a series of histochemical reactions in common, it has been postulated that carcinoids take origin from enterochromaffin cells These reactions are known collectively as enterochromaffin reactions, and the substance they reveal has been termed enterochromaffin The most important reactions include the argentaffin reaction, diazo reactions and ultraviolet fluorescence A less specific reaction is Schmorl's ferricyanide method and it yields a positive outcome also with other substances capable of reducing ferricyanide to ferrocyanide, for example melanin and sulfhydryl groups (*Pearse* 1960) Strong evidence has been accumulated suggesting that the enterochromaffin substance is a condensation product of formalin and serotonin (5-hydroxytryptamine) Thus it has been demonstrated that carcinoid tumours produce and store serotonin (*Lembeck* 1953, *Pernow & Waldenstrom* 1954), that reserpin administration is accompanied by parallel reductions of the serotonin quantity and enterochromaffin reactivity in the intestines of some animal species (*Benditt & Wong* 1957) and, in model experiments, that formalin treated serotonin exhibits positive enterochromaffin reactions (*Benditt & Wong* 1957, *Holcenberg & Benditt* 1961)

A variety of methods are accordingly available for demonstrating enterochromaffin substance in carcinoids, but the argentaffin reactions has become dominant A positive argentaffin reaction has been deemed essential for the diagnosis of carcinoids indeed, it has even been proposed that the term carcinoid should be replaced by "argentaffin carcinoma" (*Willis* 1960) The literature, on the other hand includes several reports of non argentaffin, intestinal and gastric carcinoids, even cases in which the tumour tissue was found to have a high serotonin content (*Thorson* 1958) Like intestinal carcinoids, bronchial carcinoids are capable of producing serotonin and its precursor 5 hy-

The investigation was supported by grants from the *Swedish Cancer Society* Diazo dyes were supplied by courtesy of *Imperial Chemical Industries Ltd Dyestuffs Branch Sweden*

reaction For unknown reasons a tumour with a positive fresh diazo reaction showed a negative reaction with Fast Garnet GBC The strength of the reactions varied widely from one part to another of the sections but were usually most marked marginally in the tumour lobes Owing to the few occasions when biochemical tests had been carried out on the patients, the strength of the reactions could not be correlated to any indications of serotonin production Interesting, however, is that the most reactive tumour was a metastasis to the liver from a primary focus of unknown site

TABLE 2

Histochemical Reactions of 27 Carcinoid Tumours

Site	No of tumours	No of tumours with positive reactions			
		Argentaffin	Ferri-cyanide	Fast red B (fresh)	Fast garnet GBC (stable)
Appendix	18	14	18	18	18
Small intestine	5	3	5	5	4
Metastases	4	4	4	4	4
Total	27	21	27	27	26

The argentaffin tumours presented a brown to black, fine-grained silver precipitate in the cytoplasm of the tumour cells, no unspecific silver precipitate could be observed in the connective tissue stroma After prolonged incubation, the non-argentaffin tumours exhibited unspecific silver deposits in the connective tissue stroma but the reaction in the tumour lobes remained negative

The diazo methods imparted an orange to red tint to the granules in the cytoplasm of the tumour cells Enterochromaffin cells were consistently coloured red Even within the same tumour some areas contained orange cells and others red cells, the latter giving the impression of being more densely granulated

Schmorl s ferricyanide method caused the tumour cells to stain deep blue

The appearance of the various reactions in a typical case is illustrated in Figs 1, 2, 3, and 4

DISCUSSION

The present investigation on a series of 27 carcinoids demon... that ont...

... diazo and ferricyanide reactions in 7 out of 11 biopsy specimen...

th

... a condensation pro-

yellow colour which is not particularly disturbing These observations are in agreement with those reported by *Lillie, Greco-Henson & Cason* (1960)

Schmorl's ferricyanide reaction was performed according to Pearce (1960)

The various methods were compared by testing them on human appendix The argentaffin, fresh diazo and ferricyanide reactions were each applied on two of six consecutive serial 4 μ sections from each of five normal human appendices in the order given in Table 1 Typical enterochromaffin cells with basal granulation were observed in the deeper layers of the glandular epithelium in all sections The total number of enterochromaffin cells in each section was counted and the results are given in Table 1 The diazo method tended to give a smaller number of cells than the argentaffin and ferricyanide method but the difference is not statistically significant

When material from carcinoid tumours was tested a section of normal human appendix was placed in each cuvette and served as a control

TABLE 1

Comparison Between the Reactions on Normal Human Appendices

Section No	Consecutive 4 μ sections					
	1	2	3	4	5	6
Entero-chromaffin cells, Mean ± S E	17 2±4 2	17 3±3 1	18 1±3 8	14 1±2 5	18 7±4 1	13 5±2 2

Treatment

Section No 1	Argentaffin reaction	Section No 4	Diazo reaction Fast Red B
Section No 2	Ferricyanide reaction	Section No 5	Ferricyanide reaction
Section No 3	Argentaffin reaction	Section No 6	Diazo reaction Fast Red B

RESULTS

In this series of 27 carcinoid tumours, 18 were located in the appendix, 5 in the small bowel, and 4 were carcinoid metastases to liver lymph node and mesentery from primary tumours of unknown site While 11 of the 18 appendix carcinoids extended to the serous coat, the rest invaded only the submucous and muscular coats In one case tumour tissue had invaded the ovary One of the appendix tumours exhibited multiple metastases to the mesentery At the operation 2 of the small-bowel carcinoids were found to have metastasized and showed tumour growth in the serous coat The remaining carcinoids of the small bowel were small and had been encountered accidentally

Histologically all the tumours exhibited a typical carcinoid structure, with closely spaced clusters of monomorphic and cytoplasmrich tumour cells separated by a sparse connective tissue stroma

Urinary 5-HIAA estimation had been performed preoperatively in five cases, the 24 hour output being abnormally high (80 5 and 41 mg) in two patients with metastasizing carcinoids of the small bowel

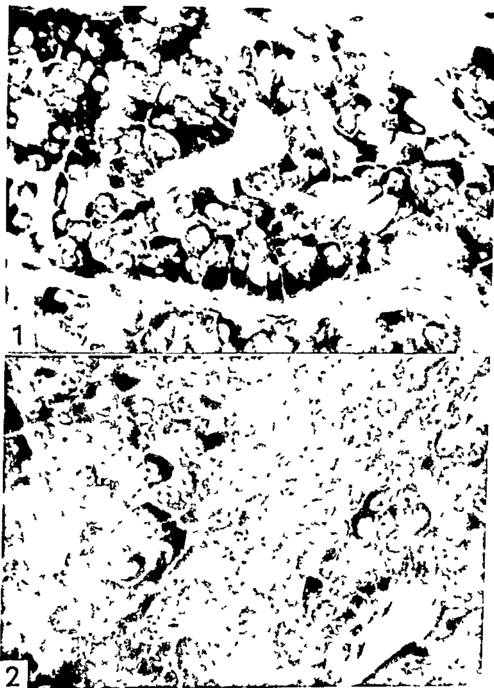
The results of the histochemical reactions are shown in Table 2 The fresh diazo reaction and the ferricyanide reaction were positive in all the tumours, while six of the tumours exhibited a negative argentaffin



Figs 3 4

Fig 3 Same tumour as in Fig 1 Schmorl's ferricyanide reaction No nuclear stain Cytoplasmic granules stained blue owing to reduction of ferricyanide to ferrocyanide Cell boundaries somewhat indistinct because of slight diffusion × 518

Fig 4 Same tumour as in Fig 1 Alkaline diazo reaction (Fast Garnet GBC) and Ehrlich's haematoxylin as nuclear stain The majority of tumour cells in this field present powder fine orange coloured granulation but the darker cells in the centre of the figure are densely granulated and have a brick red colour × 518



Figs 1 2

Fig 1 Carcinoid liver metastasis Argentaffin reaction Faintly stained nuclei (Safranin O) Black silver deposits in the tumour cells especially those located along the margins of the tumour lobes No unspecific silver deposits in the connective tissue stroma × 518

Fig 2 Same tumour as in Fig 1 Alkaline diazo reaction (Fast Garnet GBC) Weakly staining nuclei (Ehrlich's haematoxylin) Orange to brick red cytoplasmic granulation × 518

The negative argentaffin reactions, however, demand special consideration. Our series included no less than 6 out of 27 cases in which the argentaffin reaction was negative despite positive diazo and ferricyanide reactions. The precisely controlled length of the incubation period, to avoid the appearance in the sections of unspecific silver deposits, might conceivably have reduced the sensitivity of the silver impregnation method. Actually this could not have been the case for sections from all non-argentaffin tumours were reincubated until unspecific silver deposits appeared without the tumour cells themselves showing positive reactions. Furthermore, the comparison of the methods on appendix demonstrates that the argentaffin reaction is at least as sensitive as the other reactions for the purpose of visualizing enterochromaffin cells. The only plausible explanation left would seem to be that the argentaffin reaction on the one hand and the diazo and ferricyanide methods on the other have dissimilar chemical substrates, implying that they demonstrate a chemical difference between carcinoid tumour cells and enterochromaffin cells. Before any definite conclusions about this matter can be drawn, however, it will be necessary to study in greater detail the behaviour of these reactions in carcinoids, especially to compare directly the serotonin content of the tumours with the argentaffin and diazo reactions. Such investigations do not seem to have been performed, and it would probably be difficult to collect a reasonably large series in view of the fact that these studies would require the carcinoid to be diagnosed prior to extirpation of the tumour.

Thus, even if the discrepancy between the argentaffin reaction on the one hand and the diazo and ferricyanide reactions on the other lacks a satisfactory explanation at present, the results of the present investigation seem to indicate that the argentaffin reaction is *not alone* an adequate procedure for the histochemical determination of enterochromaffin material in carcinoids. The method should be combined with diazo reactions *and* the ferricyanide procedure. Besides, the argentaffin reaction is technically complicated and requires considerable experience. It can probably be dispensed with altogether for routine use while the diazo and ferricyanide procedures would seem to lend themselves well to incorporation into the ordinary programme of laboratory tests.

SUMMARY

In a series of formalin fixed biopsy specimens from 27 carcinoid tumours alkaline diazo reactions and Schmorl's ferricyanide reaction were positive in all cases, although the argentaffin reaction was negative in 6 out of the 27 cases. All reactions were compared with respect to their ability to visualize enterochromaffin cells in h

duct of a 5-hydroxyindole and formalin. Studies on gelatin models by *Benditt & Wong* (1957) disclosed that the methods adopted here are capable of demonstrating the presence of serotonin in concentrations as low as 1 mg per ml. Negative histochemical reactions would in other words imply a serotonin content below this threshold of sensitivity. The strength of the reactions varied widely in our series, from cases in which practically all tumour cells contained positively reacting granules to cases in which only small areas of the tumours exhibited positive material. This phenomenon, combined with reports of negative enterochromaffin reactions in carcinoids, could mean that the serotonin content of carcinoids varies strongly. There are other reasons for assuming that this is so. In patients with carcinoid syndrome the manifestations believed to be associated with rises in the serotonin level of the blood occur paroxysmatically and the urinary excretion of serotonin metabolites varies greatly from day to day, suggesting that the serotonin production in the tumours is characterized by alternating phases of storage and maximal secretion. Moreover, direct serotonin analysis in carcinoids have also yielded very variable values. Thus, *Davies* (1962) found a serotonin content varying from 1.2 to 1500 μg per gramme of tumour tissue. The former value applied to a carcinoid metastasis to the liver and does not exceed the serotonin content of normal liver tissue.

In this connexion the possibility should be taken into consideration that the histological preparation procedure might release serotonin from the tumours. Serotonin might be present in the tumour cells in a readily soluble form, but the condensation product of serotonin and formalin has low solubility in water and the processing media employed in paraffin techniques. Accordingly dissolution could take place during formalin fixation. A recently advanced histochemical procedure for localization of monoamines on the cellular level with the aid of freeze drying and formalin-vapour fixation (*Falck* 1962) demonstrates, among other things, fluorescence in mast cells of the same type as in enterochromaffin cells. After fixation in an aqueous solution of formalin and routine paraffin embedding mast cells usually exhibit no such fluorescence, indicating that dissolution of reactive material can take place during the ordinary histological processing, at least from mast cells.

Clearly, therefore, enterochromaffin material demonstrated by regular methods may vary owing to technical as well as biological factors. Consequently negative histochemical reactions cannot rule out a diagnosis of carcinoid tumour in cases in which the morphological picture is not typical. Conversely, the results of the present investigation show that the serotonin quantity in carcinoids in practice rarely lies below the sensitivity thresholds of the diazo methods and Schmorl's ferricyanide procedure after routine formalin fixation. Hence these methods are evidently of great value in the diagnosis of carcinoid tumours.

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The Swiss Tropical Institute (Head Professor R Geigy), the Outward Clinic of the University of Basle (Head Professor O Gsell) and the University Department of Pathology General Hospital Malmo (Head Professor J Linell)

MAST CELLS AND CORONARY HEART DISEASE[1]

Relationship between Number of Mast Cells in the Myocardium, Severity of Coronary Atherosclerosis and Myocardial Infarction in an Autopsy Series of 672 Cases

By

MICHEL FERNEX and NILS H STERNBY

Received 23 iv 64

As many clinical and experimental studies have shown a possible relationship between mast cells and atherosclerosis we thought it worth while to study the relation between coronary atherosclerosis respectively myocardial infarction and the number of mast cells of the myocardium in a large autopsy series from a well defined population

Mast cells are important elements of the connective tissue They vary in shape they have a round central nucleus and their cytoplasm contains basophil granules

Mast cells are said to be the chemical laboratory of connective tissue and to play an important rôle in the turn over growth and repair of this tissue

Atherosclerosis is a degenerative disease of the artery wall associated with changes in lipid metabolism and, perhaps in the blood clotting system A common

this complex clearing factor or lipoprotein lipase but rather to a deficiency in the releasing mechanism of this enzyme since longterm parenteral treatment with heparin of hyperlipaemic patients does not produce any signs of exhaustion of the

[1] This work was supported by a grant of *The World Health Organization* section for *Cardiovascular Diseases* and by *The Swiss National Fund for Scientifical Research* The vessels weere collected in a study supported by *The Swedish National Association against Heart and Chest Diseases*

it appeared that the use of elevated temperature during incubation in the silver solution necessitated precise control of the duration of the incubation period *to avoid unspecific silver deposition* Carcinoids showing a negative argentaffin reaction were reincubated until an unspecific silver deposit appeared in the section but the tumour cells still remained negative In human appendix, on the other hand, the argentaffin reaction visualized at least as many enterochromaffin cells as the other methods The reason for these discrepancies in the argentaffin reaction between enterochromaffin cells and carcinoid tumour cells is obscure, but their mere existence implies that the argentaffin reaction alone is of limited value in the diagnosis of carcinoids

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Mast cells are important elements of the connective tissue They vary in shape they have a round central nucleus and their cytoplasm contains basophil granules

* Wilander concentra apart from

blood basophils are the only cells known to produce this mucopolysaccharide (3 9) The function of the mast cells however is complex They produce also histamine (37 57 61) and their cytoplasm shows a high enzymatic activity (4 14 16 44 60)

hyperlipaemia does not appear to be the consequence of a lack of this complex clearing factor or lipoprotein lipase but rather to a deficiency in the releasing mechanism of this enzyme Since longterm parenteral treatment with heparin of hyperlipaemic patients does not produce any signs of exhaustion of the lipoprotein lipase Endogenous heparin might be the deficient releasing agent (19 64)

As mast cells produce endogenous heparin many authors have d

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This might however, be the way to show the role played by the heparinocytes in the development of atherosclerotic diseases A similar approach to this problem is counting of tissue mast cells

Cairns & Constantinides noted that experimental atherosclerosis was easy to induce in animals with few mast cells such as rabbits but not in rats which have a large number of tissue mast cells They also studied these cells in sections (7 µ) of the myocardium of man 60–90 years of age They found fewer mast cells in cases with than in those without advanced atherosclerotic changes in the coronaries (10 11 12) *Mills Strickland & Paterson* later showed that the mast cell number in man remained unchanged until 24 hours after death (43) They noticed that the mast cell number was lower in cases with than in those without stenosis or thrombosis of the coronary arteries (50)

In some other studies the mast cells within the walls of more or less atherosclerotic arteries have been counted and seemed to be more numerous in diseased than in normal vessels This localized mastocytosis might however be related to the local *inflammatory process in the vessels where the number of lymphocytes is* also increased (51 52)

The number of mast cell decreases at one and the same rate with age in human skin myocardium (33) and the adventitia of veins (68) On the other hand atherosclerosis increases with age In African people where serum cholesterol values are low and coronary heart disease practically nonexistent the mast cells in the skin and myocardium are approximately twice as abundant as in Europeans where the incidence of myocardial infarction is high (21 23) It has also been shown that hypercholesterolaemia is common in generalized urticaria pigmentosa a diffuse mast cell proliferation (24 54 62)

Lempert Stein & Doyle found the number of mast cells in the myocardium to differ from one part of the muscle to the other and therefore concluded that the counting of mast cells in a single or a few specimens was not reliable (39a) On the other hand in a larger series *Hellstrom & Holmgren* found a good correlation between different parts of the myocardium and also in different pieces of skin (33)

Many authors have stressed the role of mast cells in experimental atherosclerosis (11 13 27, 28 70) *Watson* could not induce atherosclerosis by the use of a high fat diet in rats treated with histamine liberators (71) substances which cause mast cells to disrupt (20 49 57 59) These mast cells are not destroyed but difficult to recognize on histologic slides (30) Also *Douglas et al* failed to induce experimental atherosclerosis by this method (13) Atherosclerosis could however be produced in the rat by combining a fatty diet and thiouracil which decreases the mast cell number and reduces their activity (25)

P Fernex used worm infections and histamine liberators to stimulate mast cells in rats and noted an acceleration of catabolism of alimentary fats in rats with previously stimulated mast cells (26)

MATERIAL AND METHODS

The material was collected from 794 consecutive autopsies from cases aged 18 to 98 years performed at the General Hospital Malmö Sweden The hospital serves a city with about a quarter of a million inhabitants About 60 per cent of all deaths within the city occur in the hospital and the autopsy rate is 99 per cent Most of the autopsy cases were above 65 years of age and no legal cases were included The autopsies were performed 2 48 hours after death mostly within the first 12 18 hours

The coronary arteries were opened longitudinally by the prosector and then dissected from the heart by technicians The vessels were cleaned from fat fixed in 10 per cent neutral formalin for 12 24 hours and placed in transparent plastic bags with a small amount of formalin according to a method described by *Holman et al* (34) With the specimens preserved in this way evaluation could be postponed until all the specimens had been collected

The main trunk of each of the coronary arteries was graded separately according to the following scale

Grade 0 no lesions or a few lipid streaks
Grade 1 numerous lipid streaks with or without a few fibrous plaques
Grade 2 numerous fibrous plaques with or without very small calcifications but without any complicated lesions (bleedings ulcerations and thrombi)

Grade 3 small to fairly extensive calcifications with or without small complicated lesions

Grade 4 extensive calcified and/or complicated lesions

For each case the sum of the three grading figures was calculated, and the cases were than divided into three types of coronary atherosclerosis according to the following scale

Grading sum 0 3 *slight* coronary atherosclerosis
Grading sum 4–6 *moderate* coronary atherosclerosis
Grading sum 7–12 *severe* coronary atherosclerosis

For mast cell counting in the myocardium specimens were taken at the base of the front and the lateral wall of the left ventricle one piece from each ...

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tolu ... mast cells which appeared dark violet on a very light blue field were counted in 40 adjacent fields measuring 1/4 mm² each in both pieces and the average value was reported per square millimeter myocardium

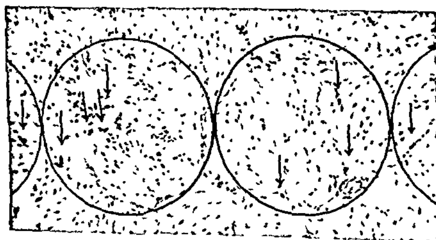


Fig 1

The appearance of mast cells in the myocardium (Toluidine Blue 75 ×)

192 cases were excluded for various reasons

in 64 cases pieces of myocardium were missing or consisted of only necrotic or granulomatous connective tissue

in 23 cases macroscopic description of the myocardium or other important pathological data were missing

in 29 cases ...
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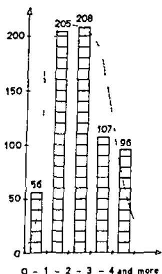
RESULTS

Number of Mast Cells in the myocardium

The number of mast cells ranged between 0.1 and 11.5 per mm² of myocardium. The number of cases in different age groups and in 5 groups according to the mast cell number is given in Table 1.

TABLE 1
Distribution of 672 Cases According to Age and to Mast Cell Number in the Myocardium

Mast cells mm²	0-1		1-2		2-3		3-4		>4		Total number of cases
Age groups	Number	%	Number	%	Number	%	Number	%	Number	%	
18–60	12	*21*	32	*16*	40	*19*	17	*16*	33	*34*	134
61–70	17	*30*	48	*23*	47	*23*	31	*29*	22	*23*	165
71–80	21	*38*	74	*36*	67	*32*	37	*34*	28	*29*	227
>80	6	*11*	51	25	54	26	22	*21*	13	*14*	146
All ages	56	*100*	205	*100*	208	*100*	107	*100*	96	*100*	672



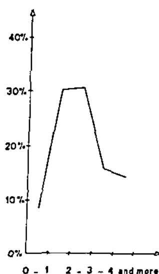


Fig. 2

Distribution of 672 cases according to their mast cell number. To the left: absolute number of cases, to the right: percentual distribution.

In 61 per cent of the cases there was between 1 and 3 MC/mm². In about 8 per cent, less than 1 MC/mm² was found. Fig. 2 shows the distribution of all the cases according to mast cell number. The cases with less than 1 MC/mm² are said to have *"few mast cells"*, the cases with

1–3 MC/mm² are said to have a *normal mast cell count* and the cases with more than 3 MC/mm² are said to have a *large number of mast cells* in their myocardium

Mast Cell Number in Different Age Groups

In all age groups cases with a large or small mast cell number were encountered (Table 1) However the youngest age group which represents only 20 per cent of the cases includes 35 per cent of the cases with more than 3 MC/mm² In the older age groups the proportion of cases with a high mast cell count is decreasing (Fig 3)

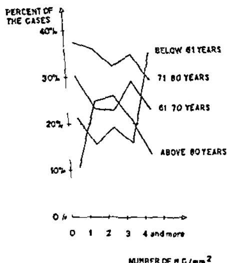


Fig 3

Percentual distribution of the cases in different age groups according to the mast cell number

Mast Cell Number According to Sex

In this autopsy series the sex ratio was nearly 1/1 (49 per cent women) No sex difference in mast cell number was found (Fig 4)

There were only 28 women below 50 years of age of whom 12 can be considered as premenopausal (neither castrated nor receiving massive sex hormone therapy) The average mast cell number in these 12 cases was 2 86 per mm

Mast Cells and Severity of Coronary Atherosclerosis

Atherosclerotic changes in the coronary arteries were graded as slight moderate or severe The percentage of cases with slight coronary atherosclerosis decreased with age 56 per cent of the cases less than 61 years

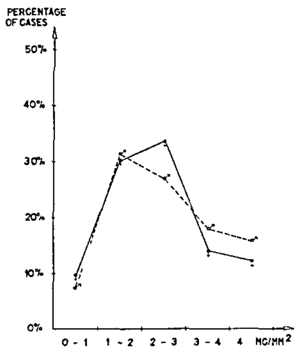


Fig 4

Distribution of mast cell numbers among men and women

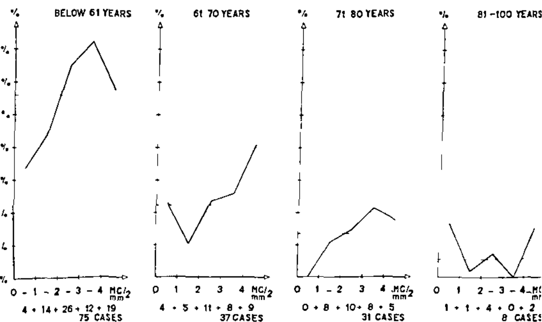


Fig 5

Distribution of cases with slight coronary atherosclerosis according to age and mast cell number

f age 22 per cent of those between 61 and 70, 14 per cent between 71 nd 80 and only 6 per cent more than 80 years of age However, the ercentages were different in most age groups when the number of mast ells was considered Fig 5 shows that a high percentage of cases with light coronary atherosclerosis occurs not only in younger age groups, ut also in subjects with a high mast cell count This holds for ages up o 80 but in the oldest age group, only very few coronaries remain free rom severe atherosclerotic changes

Fig 6 shows the percentual distribution of the cases with slight, moderate and severe coronary atheroclerosis in relation to mast cell number It is obvious that the cases with slight atherosclerotic changes often have a high mast cell count and conversely, that the cases with severe atheromatosis often have a low mast cell count

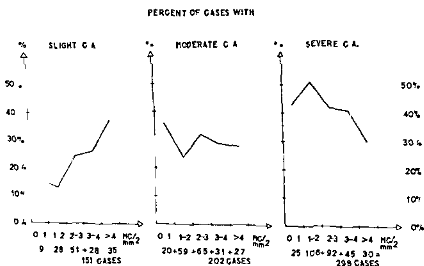


Fig 6

The relation between the severity of coronary atherosclerosis and mast cell number

Incidence of Myocardial Infarction According to Age, Sex and Mast Cell Number

Myocardial infarction both recent and old was recorded in 35 6 per cent of the 672 autopsies It occurred most frequently between 61 and 70 years of age where the incidence was 45 per cent

Of 151 cases with slight coronary atherosclerosis 6 per cent showed myocardial infarctions In 202 cases with moderate coronary athero sclerosis the inci[illegible] was 23 5 per cent and in 298 cases wi[illegible] it was 56 7 per cent The cases wi[illegible] ot equally distributed when related to the number of mast cells in the left ventricle 46 per

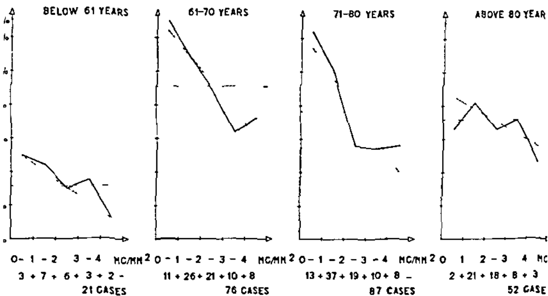


Fig 7

Incidence of myocardial infarctions in different age groups according to mast cell number The dotted line represents the regression line

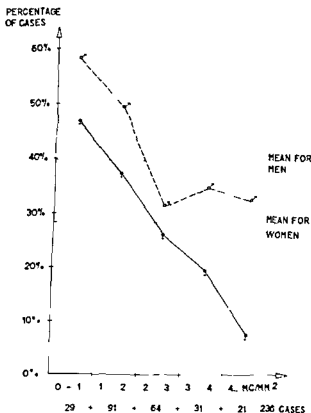


Fig 8

Incidence of myocardial infarction according to mast cell number in men and women

cent of the cases with a low mast cell count (< 2 MC/mm²), showed infarction, against 30 per cent of the cases with a "normal" mast cell count (2–3 MC/mm²) and only 24 per cent of the cases with a high mast cell count (> 3 MC/mm²) So the incidence of myocardial infarction is distinctly higher in cases with a low than in cases with a high mast cell number This holds for all age groups (Fig 7) The calculation of the regression line[1] for the different age groups showed that the difference in the incidence of myocardial infarction in individuals with more or fewer mast cells is highly significant for the age groups between 61 and 80, and nearly significant for the youngest group It is not significant for the oldest age group

The incidence of myocardial infarction was higher in men (39 8 per cent) than in women (28 3 per cent) in this series In both sexes the incidence of myocardial infarction decreases with increasing number of mast cells (Fig 8)

Of 672 cases 96 had more than 4 MC/mm² Myocardial infarctions were recorded in 21 (21 8 per cent) of these cases, 3 females and 18 males In men with a high mast cell count, the incidence of myocardial infarction thus remains fairly high Among the men was one case of essential hypercholesterinaemia and one of uric diathesis These two diseases are inborn errors of metabolism frequently associated with coronary occlusion but the pathogenesis of coronary occlusion in these cases might be different from that in ordinary atherosclerotic disease In 3 cases the cause of death was syphilitic aortitis, where the ischaemic heart disease was not due to coronary atherosclerosis Eight died from different malignant diseases

DISCUSSION

The number of mast cells in the myocardium from the left ventricular wall was found to vary between 0 1 and 11 5 *per mm²* These values are about the same as those found in other white populations, *e g* from Canada (10) and Paris (21), but much lower than those in the Senegalese population of Dakar (21) For an ' European Standard ', one might consider 0–1 MC/mm² as a low mast cell count, 1–3 MC/mm² as a normal, and above 3 MC/mm² as a high mast cell value

For African populations, where the average value is 5 2 MC/mm², a high mast cell count starts at 6 MC/mm²

Pr[illegible]

my[illegible]

dec[illegible] rats (2, 12, 72) In mice, however, an increase in the mast cell number with age has been reported (67)

In our material no cases below 18 years of age were included No significant difference in myocardial mast cell number appeared in the

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[1] We are grateful to Prof F Linder Geneva for the statistical analysis (40)

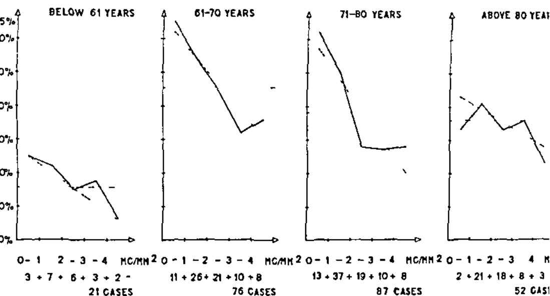


Fig 7

Incidence of myocardial infarctions in different age groups according to mast cell number The dotted line represents the regression line

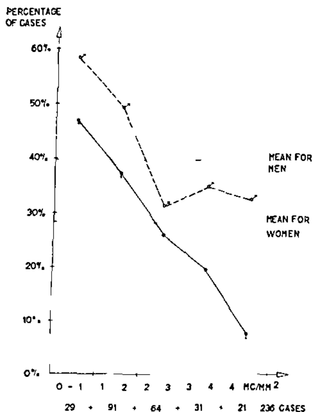


Fig 8

Incidence of myocardial infarction according to mast cell number in men and women

The reduced incidence of myocardial infarction in subjects with many mast cells in the myocardium might be related to the activities of these cells If so it might be possible to reveal some "predisposing factor for atherosclerosis by measuring the excretion of histamine and or heparin metabolites in urine the variation in mast cell specific enzymatic activity in blood or the mast cell number in a standard skin biopsy since the number of mast cells in myocardium and skin vary in the same way (33)

SUMMARY

In a large autopsy series from Sweden the relationship between coronary atherosclerosis myocardial infarction and mast cell number of the myocardium was studied The number of mast cells was found to be the same as in other white populations

Cases below 80 years of age with a high mast cell number showed less coronary atherosclerosis than cases with a low number of mast cells

The incidence of myocardial infarction recent and old in the age group 61 80 years (392 cases) was significantly lower for cases with a high mast cell count than for cases with few mast cells

The relationship between the function of the mast cells and athero sclerosis is discussed

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different age groups, although individuals with high mast cell count were more frequently found among younger people In previous investigations sex differences in the mast cell number have been noted in individuals below 40 years of age (33) This age group is very small in our material The twelve premenopausal women have about the same number of mast cells as the rest of the population

A relationship was found between grade of coronary atherosclerosis and mast cell number in people less than 81 years of age Subjects with higher mast cell count appeared to show less coronary atherosclerosis

A statistically highly significant relation was also found between number of mast cells and incidence of myocardial infarction in the 392 subjects aged 61–80 years Infarction occurred more frequently in cases with a low mast cell count A similar result, but not statistically significant, was found in 134 cases below 60 years In the 146 cases above 80 years a similar tendency was still demonstrated 23 cases with myocardial infarction had more than 4 MC/mm² This group included one case of essential hypercholesterolaemia with xanthomatosis, one case of uric diathesis and 3 cases of syphilitic aortitis in which the pathogenesis of coronary occlusion differs from that of ordinary coronary heart disease In hypercholesterolaemia with xanthomatosis, the extremely high mast cell number might be interpreted as a reactive proliferation of mast cells to this dyslipaemia The mast cells do not, however, succeed in influencing the lipid metabolism because heparin fails to reduce the hypercholesterolaemia (63)

Many authors accept that the disturbance in the lipid metabolism in coronary heart disease might be partly related to a reduced heparin production (19, 46, 66) A hypoplasia of the mast cell system could be the cause of a hypoheparinaemia

Does the change in number of mast cells precede the atherosclerosis of the coronary arteries or is it the consequence of ischaemia or any other disturbance? Observations on the degree of coronary atherosclerosis in Africans, where myocardial infarction and angina pectoris is practically non-existant, the low serum cholesterin level in patients with diffuse idiopathic mastocytosis (24) and the parallelism of the decrease of the mast cell number in human skin and heart with ageing (33) argues for the first hypothesis, as do observations in laboratory animals where the reduction of the mast cell activity and number preceeds the vessel changes (13, 25, 64)

Mast cells thus appear to be involved in the mechanism of defence against atherosclerotic degeneration of arteries They interfere with coronary heart disease in two ways In producing heparin mast cells accelerate the catabolism of the alimentary lipids and consequently increase the fibrinolytic activity and reduce the clotting tendency of blood (39, 45) The production of very active enzymes and histamine is another important function Histamine released during ischaemia might prevent extensive necrosis by inducing local vasodilatation and development of collaterals

The reduced incidence of myocardial infarction in subjects with many mast cells in the myocardium might be related to the activities of these cells. If so, it might be possible to reveal some "predisposing" factor for atherosclerosis by measuring the excretion of histamine and/or heparin metabolites in urine, the variation in mast cell "specific" enzymatic activity in blood or, the mast cell number in a standard skin biopsy, since the number of mast cells in myocardium and skin vary in the same way (33).

SUMMARY

In a large autopsy series from Sweden the relationship between coronary atherosclerosis, myocardial infarction and mast cell number of the myocardium was studied. The number of mast cells was found to be the same as in other white populations.

Cases below 80 years of age with a high mast cell number showed less coronary atherosclerosis than cases with a low number of mast cells.

The incidence of myocardial infarction, recent and old, in the age group 61–80 years (392 cases) was significantly lower for cases with a high mast cell count than for cases with few mast cells.

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SARCOMAS IN ALBINO MICE INOCULATED WITH ROUS CHICKEN TUMOUR MATERIAL

By

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Rous sarcoma has been transplanted to various mammals, including mice. *Roskin* (1927) reported a fairly long temporary growth of such transplants in mice with blockaded reticulo-endothelial system. Similar results have been obtained by *Greene* (1951) and *Ageenko* (1957), who transplanted the tumour to the brain of the mouse and by *Algire et al.* (1958), who used diffusion chambers. In this laboratory subcutaneous transplants of the Rous chicken sarcoma (Mill Hill strain) to new born mice survived for a short time and showed signs of active growth during the first week (*Ahlström & Jonsson* 1962 a). Cortisone treatment did not affect the duration of survival of the transplants.

Previous experiments have thus produced no evidence of pathogenicity of the Rous sarcoma virus for mice and heterotransplants of the chicken sarcoma regressed after temporary growth. Besides this, most investigators have transplanted the tumour to immunologically shielded sites or used animals with impaired immunological reactivity.

In recent years, however, several reports on the pathogenicity and tumour producing effect of Rous sarcoma virus in mammals have appeared (*Zilber & Kryukova* 1957, *Svet-Moldavsky* 1957 and 1958, *Schmidt-Ruppin* 1959, *Svoboda & Grozdanovic* 1959, *Svoboda* 1961). *Schmidt-Ruppin* (1959) reported the development of sarcomas in heterozygous albino mice following intramuscular injection of Rous sarcoma material. The tumours could be carried in series in mice. The transplantable mouse tumour of Schmidt-Ruppin had a chromosome pattern different from that of the chicken sarcoma and similar to that usually found in mouse tumours (*Ising-Iversen* 1960). *Ising-Iversen* considered that "heterologous injection of Rous virus might have given rise to a virus induced tumour differing in chromosome pattern and histologic picture from the Rous sarcoma".

This paper deals with the results of an extended investigation on mice inoculated with the same strain of Rous sarcoma virus as that used by *Ising-Iversen*. In a large series of investigation this virus strain has been shown to produce tumours in rats, hamsters, guinea pigs and rabbits (*Ahlström et al.* 1962, 1963).

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MATERIAL AND METHODS

The Rous sarcoma material was originally obtained from Dr H Schmidt Ruppin Frankfort on the Main It has been maintained in this laboratory by serial passages in 2 to 3 week old White Leghorn chickens or in some periods kept at 65° C Chicken sarcoma tissue was finely minced with scissors and suspended in 4 parts of Hank s solution (w/v) containing 100 units of penicillin and 0 1 mg of streptomycin per ml After intramuscular injection of 0 5 ml of the suspension into the pectoral muscle of the fowl a tumour developed in a few days with the usual appearance of a Rous sarcoma The tumour was transferred at about 14 day intervals A suspension of chicken sarcoma tissue prepared in the same way was used for injection into mice

Extracts of chicken sarcoma were prepared in the following way Tumour tissue was thoroughly ground with sterile glass powder in a mortar or in a glass homogenizer and suspended in 4 parts of Hank s solution containing antibiotics as described above After clarification in an MSF Super Multex centrifuge for 15 minutes at 4 000 r p m the supernatant was centrifuged for 20 minutes at 10 000 × g at 3° C in an International Cold Centrifuge The supernatant was cautiously sucked off This procedure was repeated twice The final supernatant was used for inoculation of mice

For the preparation of Seitz filtrates the usual sarcoma suspension or homogenate was centrifuged in the MSF centrifuge at 4 000 r p m after which the supernatant was passed through a Seitz filter

The white mice belonged to a closed colony of Swiss mice which has been bred at the institute for many years They were fed standard commercial pellets with an addition of milk and fresh greens

RESULTS

1 *Inoculation of New born Mice with Minced Chicken Sarcoma*

A suspension of finely minced chicken sarcoma was injected into 21 litters of mice, 0 to 4 days of age Animals below 24 hours of age at the time of inoculation received 0 03 or 0 05 ml, the remainder 0 05 or 0 1 ml The dose was given subcutaneously on the back the needle being introduced via the left hind leg to reduce back flow Nearly half of the animals died or were lost by cannibalism as a rule within the first week of the injection

Gross findings During the first week a swelling developed on the back of some of the animals similar to that seen after injection of a suspension of Rous sarcoma of the Mill Hill strain (*Ahlstrom & Jonsson* 1962 a)

Of the surviving 72 animals however 23 developed progressively growing sarcomas at the site of inoculation The first tumours appeared 19 days after the inoculation while one tumour could not be palpated until nearly 9 months after inoculation Twelve of the tumours could initially be felt between the 19th and the 45th day

The tumours were circumscribed and firm initially non adherent

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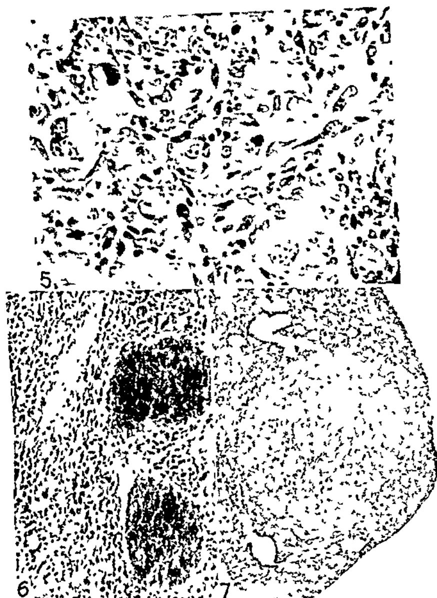
Figs 1–4

Fig 1 Sarcoma on the back of a 19 day old mouse inoculated at the age of 2 days

Fig 2 el ngated cells Htx eosin × 160

Fig 3 igl er magnification Two types of cells are seen Htx eos t

Fig 4 Network of argyrophil fil ril in the sarc ma I a dlaw × 400



Figs 5 7

Fig 5 Another part of the same tumour Large cells with abundant cytoplasm among the elongated cells Htx eosin × 400

Fig 6 Large distended vessels in the tumour tissue Htx eosin × 160

Fig 7 Lung metastasis in a mouse Htx eosin × 63

but later adherent to the musculature and the spine They grew rapidly and within a few weeks they often involved the back and flanks of the host The skin was often adherent to the surface of the tumour, but necrosis and ulceration were rare In some animals small lumps, apparently along the needle track, could be felt caudal to the main tumour

The animals gradually became severely disabled by the tumours and had to be killed Necropsy showed the tumours to have infiltrated the spine, the bony pelvis or femur, the musculature of the back and the flanks as well as the thoracic wall with destruction of the latter and sometimes with involvement of the lungs (Fig 1) In some animals the kidneys and adrenals were surrounded by tumour masses bulging into the abdominal cavity, no ascites was, however, seen The surface of the tumour was often smooth, polycyclic, and adjacent the subcutaneous tissue was oedematous and in some cases showed streaky confluent haemorrhages

The tumours were relatively firm in the peripheral parts, while in the central parts they were soft The cut surface was grey, moistened and lustrous The larger tumours sometimes showed irregular areas of necrosis filled with blood

Four mice showed small to peppercorn-sized firm, white tumour nodules on the surface of the visceral pleurae and in the lung parenchyma The retroperitoneal lymph nodes were often swollen but apparently not invaded by the tumour One mouse showed a peppercorn-sized tumour situated at the renal hilus and encroaching upon the ureter No metastases were seen in other organs

No cysts of the type described previously in rats and rabbits were seen

Microscopical findings The tumours largely resembled spindle-cell sarcomas and were built up of three types of cells The picture was dominated by crowded, spindle-shaped cells fairly rich in eosinophilic cytoplasm with rather large, oval nuclei with a vesicular chromatin structure and one or more large nucleoli Less numerous, intermingled *smaller cells with indistinct cytoplasm and small round or oval nuclei* fairly rich in chromatin were also seen (Figs 2–3) Gracile connective tissue fibrils surrounding small groups of tumour cells were observed (Fig 4) in sections stained with van Gieson and in sections impregnated with silver according to Laidlaw The softer parts of the tumours also contained numerous giant cells with voluminous cytoplasm, often with an eosinophil, homogeneous central portion and a more basophilic, vacuolated or streaky periphery The cells showed one or more peripheral nuclei with a vesicular structure and large nucleoli (Fig 5) No cross striation could be observed Staining for fat proved negative The examination often gave the impression of a gradual transition between different types of cells Centrally the larger tumours showed oedematous or reticular loosening and large or small irregular necrotic foci No cysts could be observed, but tissues *around and in the tumour some-*

and grew progressively invading the thoracic wall and abdominal musculature. Histologically it was identical with the tumours described above. No gross or microscopic metastases were found. In the other animal the tumour appeared 6 months after inoculation; it grew slowly and had the histological character of a highly differentiated fibrosarcoma.

In addition a number of animals were inoculated with filtrates from the chicken sarcoma prepared in different ways. Two litters of eleven 2 day old mice were inoculated with filtrate prepared by centrifugation of a suspension of chicken tumour in an MSE centrifuge twice for 10 minutes at 4 000 r.p.m. after which the supernatant was filtered through a membrane filter AF 250 (Göttingen) (pore size 1–3 μ). Eight months later a hazel nut sized tumour with the histological characteristics of an adenocarcinoma developed in the right axilla of one of the animals. It had probably originated from the sweat glands or mammae. Attempts to transfer the tumour to chicken proved negative.

A litter of six 1 day old mice were inoculated daily with 0.03 ml of the same type of filtrate on 4 consecutive days and then with 0.1 ml on 2 days. No tumour developed (observation period 13 months).

Four litters of altogether 29 animals aged 12 hours to 4 days were inoculated with filtrate prepared in the above mentioned way with chicken tumour as starting material. This tumour had developed following injection of sarcoma suspension from a mouse inoculated with chicken tumour. Nineteen animals survived; in 3 of them tumours appeared in the thigh and in the back within 8–13 months and gradually assumed the size of walnuts and metastasized to the lungs and pleurae. All the tumours had the histological character of an adenocarcinoma of the same type as that just mentioned. Inoculation of mice and chickens with these tumours gave negative results.

Two litters of 12 animals (12 hours respectively 4 days old) were inoculated with filtrate prepared in the following way. The supernatant of chicken fibroblast cultures inoculated with high titred virus pool (titre > 10 000 m.i.d./ml) and showing widespread foci were centrifuged twice at 3 000 r.p.m. in an MSE centrifuge after which the supernatant was filtered through Cellafilter grob (Göttingen) (pore size 0.6–0.8 μ). All these animals survived and an adenocarcinoma of the type described above occurred in one of them.

Altogether 93 mice aged 12 hours to 7 days were inoculated subcutaneously with Seitz filtrate of a suspension of chicken tumours. In some cases the tissue material was frozen and thawed before filtration. 81 animals survived. No tumour was found in any of them.

4. *Serial Transfer of the Sarcomas in Mice*

The mouse tumours were successfully transplanted to adult mice belonging to the same colony. Minced mouse tumour tissue was suspended in 4 parts of Hank's solution and 0.25 ml of the suspension

times contained fairly broad, bloodfilled vascular spaces lined with a hyperplastic endothelium (Fig 6) In the periphery the tumours infiltrated the fatty tissue, musculature and the skeleton In several areas mitoses were fairly numerous

The pulmonary metastases showed the same picture of spindle-cell sarcoma as the tumours at the site of inoculation (Fig 7) Some were of large-cell type, others of small-cell type The tumour metastases often formed perivascular cuffs around medium-sized vessels (Fig 8) An admixture of leucocytes was often seen in and around metastases Many animals had widespread pneumonic changes in the lungs

The tumour at the renal hilus had the character of a fairly highly differentiated fibrosarcoma This tumour might have been spontaneous Unfortunately no material from this tumour was set aside for further investigations

No metastases could be demonstrated in the retroperitoneal axillary, hilar or inguinal lymph nodes The lymph nodes often showed signs of non-specific inflammation

No metastases were seen in the liver or the spleen

2 *Effect of Age of the Mice*

The effect of the age of the mice is apparent from Table 1

The mice thus appeared to be susceptible only when inoculated within the first 2 days of life One animal developed a tumour after inoculation at 10 days of age Animals above 2 days were otherwise insusceptible

TABLE 1

Effect of Age of the Mice

Age at time of inoculation	Dose (ml suspension)	Mice with tumours / surviving mice
12 hours	0.03–0.05	12/28
1 day	0.05	3/22
2 days	0.05–0.1	6/13
3–4 days	0.05–0.1	0/6
10 days	0.15	1/10
13–20 days	0.1–0.2	0/12
adult	0.25	0/15

3 *Results of Injections of Chicken Tumour Extracts and Filtrates into Mice*

Extracts of the chicken sarcoma prepared as described above were injected subcutaneously into 6 litters of altogether 39 mice aged 12 hours to 4 days The dose injected varied between 0.05 and 0.15 ml according to the age of the animal *Of these animals, of which 2 were* lost by cannibalism, 2 developed tumours In one of the animals the tumour appeared at the site of inoculation after five and a half months

and grew progressively invading the thoracic wall and abdominal musculature. Histologically it was identical with the tumours described above. No gross or microscopic metastases were found. In the other animal the tumour appeared 6 months after inoculation; it grew slowly and had the histological character of a highly differentiated fibrosarcoma.

In addition a number of animals were inoculated with filtrates from the chicken sarcoma prepared in different ways. Two litters of eleven 2 day old mice were inoculated with filtrate prepared by centrifugation of a suspension of chicken tumour in an MSE centrifuge twice for 10 minutes at 4 000 r.p.m., after which the supernatant was filtered through a membrane filter AF 250 (Göttingen) (pore size 1–3 μ). Eight months later a hazel-nut sized tumour with the histological characteristics of an adenocarcinoma developed in the right axilla of one of the animals. It had probably originated from the sweat glands or mammae. Attempts to transfer the tumour to chicken proved negative.

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Altogether 93 mice aged 12 hours to 7 days were inoculated subcutaneously with Seitz filtrate of a suspension of chicken tumours. In some cases the tissue material was frozen and thawed before filtration. 81 animals survived. No tumour was found in any of them.

4. *Serial Transfer of the Sarcomas in Mice*

The mouse tumours were successfully transplanted to adult mice belonging to the same colony. Minced mouse tumour tissue was suspended in 4 parts of Hank's solution and 0.25 ml of the suspension

was given subcutaneously on the back to 5 adult animals. In 2 of them small nodules were palpated after 6 days, but they disappeared after a further few days. In 2 other mice, however, progressively growing sarcomas developed which were palpated on the 9th and 18th day, respectively, after the inoculation (Fig. 9). The sarcoma has since been transplanted through adult mice (60 passages). From the 5th passage the frequency of takes was, with but few exceptions, 100 per cent. The latency period decreased from 14–18 days in the first generations, to 7–10 days in the later. The survival period also decreased somewhat. The gross as well as the microscopical appearance of the transplanted sarcomas resembled that of the primarily induced tumours, though the large cell types were somewhat more numerous than in the latter (Fig. 10). In only one animal belonging to the second passage were metastases to the peritoneum seen in the form of tumour nodules on the serosal surface of the spleen, no metastases were afterwards seen in adult animals. On the other hand, metastases were found in a few animals belonging to the 3rd and 7th passages, which were inoculated at the age of 4 days or 1 day respectively. The metastases were situated in the pleura, viscera or mesenteries (Figs. 11–12).

Extract was prepared from mouse tumours in the 3rd, respectively 9th passages, in the same way as that from chicken tumours and was injected subcutaneously into all together 42 mice aged 1–10 days in doses of 0.1 or 0.2 ml. In none of these animals did tumour develop.

Seitz-filtrates were prepared from 2 freshly induced mouse tumours and 0.1 ml was injected into each of 10 one-day-old mice and 10 three-day-old mice. Ten adult mice were inoculated with 0.5 ml. Three of the young mice and 8 of the old ones survived the observation period (5 months). No tumours developed in any of them.

5. *Back Transfer of the Mouse Sarcoma to Chicken*

From each of the tumours in 3 of the 23 mice with sarcoma after inoculation with minced chicken tumour material a suspension was prepared in 5 parts of Hank's solution. Two of the suspensions were used for inoculation of 1.0 ml into the pectoral muscle of 2 roughly 3-week-old chickens and one for inoculation of a similar dose into the peritoneum. Four weeks after inoculation fairly firm pea sized tumours

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Figs. 8–11

Fig. 8. Another lung metastasis showing the perivascular distribution. Htx eosin × 160.

Fig. 9. [illegible] adult [illegible]

Fig. 10. [illegible] with dominance of large [illegible]

Fig. 11. Local tumour, bulging into abdominal cavity and metastases in mesenterium of small intestine in a mouse inoculated with mouse tumour at the age of 4 days (third passage).


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Seitz-filtrates were prepared from 2 freshly induced mouse tumours and 0 1 ml was injected into each of 10 one-day-old mice and 10 three-day-old mice Ten adult mice were inoculated with 0 5 ml Three of the young mice and 8 of the old ones survived the observation period (5 months) No tumours developed in any of them

5 *Back Transfer of the Mouse Sarcoma to Chicken*

From each of the tumours in 3 of the 23 mice with sarcoma after inoculation with minced chicken tumour material a suspension was prepared in 5 parts of Hank's solution Two of the suspensions were used for inoculation of 1 0 ml into the pectoral muscle of 2 roughly 3-week-old chickens and one for inoculation of a similar dose into the peritoneum Four weeks after inoculation fairly firm pea-sized tumours

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Figs 8 11

Fig 8 Another lung metastasis showing the perivascular distribution Htx eosin × 160

Fig 9 First passage tumour in a mouse inoculated as adult

Fig 10 (33rd) with dominance of large

Fig 11 and metastases in mesenterium ith mouse tumour at the age of of small inte ... 4 days (third passage)

were palpable in the breast musculature of the first 2 birds. The tumours grew slowly and at necropsy 4 weeks later large tumour conglomerates were seen in the thoracic wall. The tumour tissues were grey white, sometimes mat and sometimes with a lustrous cut surface resembling that of a Rous sarcoma. The microscopical picture agreed also with that of the classical Rous sarcoma with bundles of spindle shaped cells separated by a myxomatous substance and free macrophage like cells (Fig. 13). The chicken inoculated intraperitoneally died spontaneously after about 2 months with large intraperitoneal tumour masses resembling Rous sarcoma.

The tumour suspension prepared from a tumour in the first mouse passage induced tumour growth in one of the two chickens inoculated intramuscularly. Material from 15 different mouse passages was then injected into chickens, the last injection being from the 52nd passage and gave rise to tumours in most birds (Fig. 14).

Five inoculated chickens (from 4th, 7th, 9th, 12th and 20th mouse passages) showed no tumour growth, probably because the material had been taken from necrotic parts of the mouse tumour or because the chickens differed in susceptibility.

A cell free preparation of the mouse tumour was prepared by homogenizing tumour tissue in an Ultra Turrax (type 18/2, 24 000 r.p.m.) for 10 half minute periods in an ice bath and collecting the supernatant after centrifuging in the Cold Centrifuge at 10 000 g for 20 minutes. Six chickens were inoculated into the breast muscle and 4 others with a similar preparation from mouse tumour grown in tissue culture for 9 days. No tumour developed.

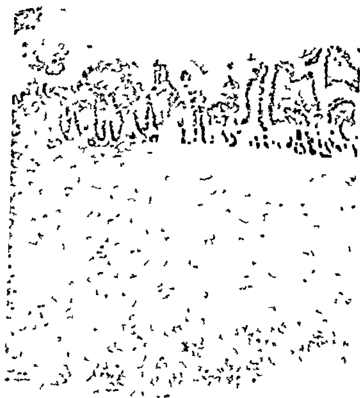
Altogether 5 chickens were inoculated with Seitz filtrate from freshly induced mouse tumours and from first passage tumours. In none of the birds did sarcoma appear.

Cell suspensions from the 2 mouse tumours observed after inoculation of chicken sarcoma extract were also inoculated into chickens. The one resembling the usual mouse sarcoma gave positive result, while material from the fibrosarcoma proved negative. The latter tumour might thus have been a spontaneous one.

6. *Occurrence of Haemagglutinins in the Tumours and of Haemagglutination Inhibition in the Sera*

In view of the possibility of contamination with polyoma virus, the tumour tissue was tested for haemagglutinins. Material from 2 of the sarcomas induced in mice was tested for haemagglutinins in the way described by *Ahlström & Jonsson* (1962 b) for the rat tumour material. No haemagglutinins were demonstrable. The investigation was kindly performed by Dr. Sven Bergman, Department of Virology, Lund.

Four adult mice were inoculated subcutaneously once a week for 3 weeks with 0.25 ml of a chicken tumour suspension in saline (1:5).





Figs 12 13

Fig 12 Metastasis in mesenterium of small intestine in the same mouse as in Fig 11 Htx eosin × 43

Fig 13 Chicken sarcoma induced [illegible] a k[illegible] transfer of a mouse tumour (fifth passage) The usual picture of a R[illegible] sarcoma Htx eosin × 100

on each occasion One week after the third inoculation the sera from the mice were tested for polyoma antiviral antibodies by the haemagglutination inhibition test (*Eddy et al* 1958) The titres were 1 30 to 1 240 and could be regarded as too low to indicate specific antibodies The investigation was kindly performed by Dr H O Sjogren Institute for Tumour Biology Karolinska Institutet Stockholm

DISCUSSION

As in rats hamsters and guinea pigs material from the Schmidt Ruppin strain of Rous chicken sarcoma (originally deriving from the late Professor Ch Oberling Paris) induced sarcomas in mice Cell suspensions from chicken sarcoma have thus evoked sarcomas in mice in a rather high frequency and in one case did a tumour occur in a mouse after inoculation with a presumably cell free supernatant of centrifuged chicken tumour material Such material has however induced sarcomas in a much higher frequency in rats and hamsters Attempts to induce tumours with Seitz filtrates in mice and in various other mammals proved unsuccessful owing probably to loss of virus during the filtration procedure Cell free filtrates prepared in different other ways have given negative results in mice even after attempts to adapt the virus to mice or after passage in chicken fibroblast cultures Similarly prepared filtrates have produced sarcomas in rats (*Ahlstrom* 1963) The tumours observed in a few of the mice were evidently spontaneous and could not be transferred back to chicken

The chicken sarcoma thus seems to contain a virus probably a variant of the Rous sarcoma virus with oncogenic capacity in mice

This is in agreement with the findings of *Zilber* and collaborators 1962) With his strain Zilber induced not only sarcomas but also haemorrhagic cysts of the same appearance as those observed in this laboratory in rats but not in mice inoculated with the Schmidt Ruppin tumour strain The surroundings of the mouse tumours induced by this strain however often contained wide distended vascular spaces The differences can probably be explained by the use of different mouse and virus strains

That the sarcomas in mice should represent heterotransplants of chicken tumour can be ruled out by various observations Thus preliminary chromosome studies (*Levan* 1962) showed that the mouse tumours were built up of mouse cells Gel precipitation tests according to *Wadsworth* (1957) revealed no antigens common to mouse and chicken sarcoma (*Skold* 1963) The relatively long interval between inoculation and the appearance of tumours progressive growth of the induced tumours and the possibility to transfer the tumour apparently indefinitely in unconditioned hosts argue definitely for the tumours being of mouse origin These problems have been discussed in greater detail in earlier reports on experiments on the tumour inducing capacity

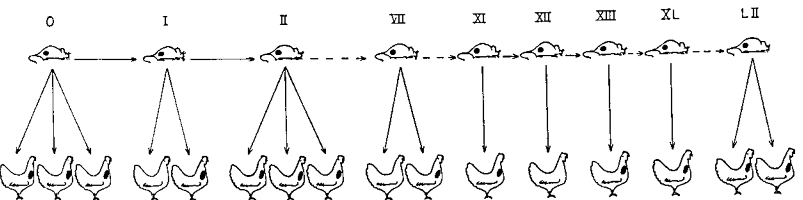


Fig 14
Diagram showing the results of back transfer to chicken of the Rous sarcoma in mice

tests, using anti-Rous sera are necessary to elucidate this problem The negative results of the tests for haemagglutinins and haemagglutination inhibition tests and the lack of cross-reactions between polyoma-induced and Rous virus induced tumours in the transplantation experiments (*Sjogren & Jonsson* 1963) argue against contamination with polyomavirus The mice inoculated with Rous virus never showed the spectrum of different tumours seen in animals inoculated with polyomavirus

Pulmonary metastases were seen in a few animals with new-induced sarcoma Whether the metastases were due to dissemination of tumour cells or to virus cannot be decided with certainty The former possibility is supported *e g* by the fact that metastases were regularly much smaller than the tumours at the site of the inoculation

The tumours could be carried in series in adult mice with cellular material already from the beginning, and from the 5th passage the frequency of takes was almost 100 per cent The mice have been kept as a closed colony for several years and are probably genetically more homogenous than the rats, in which the Rous virus induced tumours had to be carried initially in new-born animals. The adult mice, carrying passage tumours have, as a rule, shown no metastases, probably due to adaptation of the tumour to mice in which it grows quicker with consequent shorter survival of the hosts (*Ostenfeld* 1941)

All attempts to transfer the tumour from mouse to mouse with different types of acellular preparations failed even when new-born animals were used Inoculation of chickens with acellular material from mouse tumours also failed The presence of virus in the mouse tumours has, however, been proved by the production of sarcomas in chicken following injection of a cell suspension also from the late mouse tumour passages, though the relatively long latency and slow growth of subsequent chicken tumours argue for a low virus content of the inoculate (*Bryan* 1946) The virus in the mouse tumour is in some way "masked" or the virus content of the cell free preparations is too low to elicit tumours Similar results have been obtained with Rous virus induced tumours in rats, while infectious virus has been found in some hamster tumours (*Svoboda et al* 1963)

The extreme variation in virus yield from Rous chicken sarcoma has been a problem ever since the original work with this tumour, and non-infective tumours passing through "nonfilterable phases" have frequently been found One of the most important parameters determining the virus yield is the infective dose, small doses resulting in a larger fraction of non infective tumours (*Bryan et al* 1955, *Prince* 1959) This explanation, however, appears less likely since the virus doses used in the mice were large In the chicken, the virus yield does not seem to vary with the level of circulating antiviral antibodies (*Prince* 1959, *Rubin* 1962) Rubin assumes that the decrease in virus yield in the chicken tumour depends upon a cellular reaction of immunologically

of the Schmidt-Ruppin virus strain on rats, hamsters and guinea-pigs (*Ahlstrom et al* 1962 and 1963).

The findings are consistent with the susceptibility of mice to the virus being lower than that of rats, guinea-pigs and hamsters Only 30 per cent of the surviving mice, inoculated with chicken tumour suspension, developed tumours, while the remaining kinds of animals studied showed a higher frequency of takes The mice appeared mainly to be susceptible only when inoculated within the first 2 days of life, while rats developed tumours after inoculation up to the age of 23 days The latency period in the mice varied widely, probably because of variations in the absolute size of the small inocula used for the mice resulted in a much greater relative difference in the size of the dose than did the corresponding variation in the absolute volume of the larger doses used for the other mammals

The mouse thus shows the same resistance with increasing age against the oncogenic effect of the Rous virus as is known for other tumour-producing viruses, *e g* the polyoma-virus Several explanations for this phenomenon may be considered The tissues of adult animals might be resistant to tumour induction owing to an alteration in the target cells with increasing age This point will be the subject of further investigation In the polyoma system adult mice can be rendered susceptible by total body X-irradiation prior to virus inoculation (*Dawe et al* 1959), such irradiation probably interfering with the immunologic mechanism Further, the susceptibility of suckling mice could be related to an induced immunological tolerance to the Rous virus This point also requires attention To return to the polyoma-system the immunological tolerance there does not seem to be of any importance because new born mice show good antibody-forming capacity after infection with polyomavirus (*Sachs et al* 1959, *Rowe et al* 1959) although the formation of antibodies is somewhat delayed

Another observation of interest in the investigation of the resistance with increasing age is the demonstration of tumour-specific antigens in various forms of virus induced tumours, as revealed by transplantation studies The existence of antigens common to various Rous-virus induced tumours in mice has recently been reported (*Sjogren & Jonsson* 1963), even though it could not be shown whether they are identical with viral antigen(s) In analogy with the mechanism suggested by *Sjogren et al* (1961) and *Habel* (1961) for polyomavirus it is possible that Rous virus inoculated into animals induces in infected cells the development of new antigens which are foreign to the host The lack of tumour development in adult mice could then be due to the immunological response of the host animal to these antigens, while the growth of primary tumour cells in animals inoculated as new born could be explained by the development of tolerance to these antigens

The possibility of the chicken sarcoma material being contaminated with some unknown oncogenic virus must be considered Neutralization

back transfer to chicken, in which inoculation of a suspension of mou · tumour tissue resulted in a tumour with the appearance of Rous sa coma Also material from late mouse passage was effective No virus could be demonstrated in cell free preparations The state of the virus in the mouse tumour cells is discussed

The Schmidt-Ruppin strain probably represents a mutant of the Rous virus rather than an extremely virulent strain The effect in mice is in some respects different from that in other mammalian species

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competent cells in analogy with the homograft reaction to new cellular antigens, possibly identical with viral antigens Such tumour-specific cellular antigens have been shown in Rous virus induced sarcomas in mice (*Sjogren & Jonsson* 1963), and if these should be identical with viral antigens, a similar reaction in the mouse should be considered as an explanation for the lack of infectious virus in the mouse tumours Any discussion of the virus in the mouse cell cannot be more than speculative The virus could exist in the form of a provirus and the tumour cell thus be "virogenic" (*Lwoff* 1960)

Another explanation of the failure to find infectious virus in the mouse tumours can be based on *Rubin*'s (1963) conclusion that Rous sarcoma virus is a defective virus, and that non-virus-producing cells need superinfection with Rous-associated virus (RAV) for the production of infectious Rous virus The non-infective tumours in chicken after small virus doses could be explained by the solitary infection by defective Rous virus *In vitro* studies by *Temin* (1962 and 1963) with cloned strains of Rous virus have shown that infected chick cells are more likely to give rise to converted, non-virus-producing (CNVP) foci, if they are grown with mouse cells than with chick cells No virus could be found in the CNVP cells after freezing and thawing and as in the mouse tumour cells, no virus was detectable in tumour homogenates *Temin* proposed that the mammalian tumours may contain Rous virus in the same state as the CNVP cells An explanation for this state could be the unsuitable soil of the mouse cells *in vitro* and *in vivo* for the spread of RAV to the transformed and tumour cells, respectively

The negative results of inoculations of mammals with highly virulent Rous sarcoma virus strains in this and other laboratories suggest that the Schmidt-Ruppin strain is a special variant rather than an especially virulent type Preliminary serological studies point in this direction, too Decision of this point requires further serological and immunological studies

SUMMARY

After subcutaneous inoculation of a tumour suspension of Rous chicken sarcoma (Schmidt-Ruppin strain) into new born Swiss mice, 30 per cent of the surviving animals developed progressively growing sarcomas at the site of injection as a rule after 20 40 days The sarcomas were of spindle cell type with an admixture of large polygonal cells No cysts were observed A few animals had lung metastases Inoculation of cell-free filtrates gave negative results Mice 14 days of age and older were resistant

The sarcomas could be transplanted to adult mice from the beginning Metastases were, as a rule seen only after transplantation to new born animals The sarcomas could not be transferred between mice with cell-free material

The presence of virus in the mouse tumours was demonstrated by

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VARIATIONS IN THE ONCOLYTIC ACTIVITY OF HUMAN SERUM

By

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Received 14 xi 64

The lytic action of human serum on cancer cells was first described by *Freund & Kaminer* in 1910 Their observation was that while the serum of normal individuals was able to lyse human cancer cells the serum of cancer patients was often unable to do so that is to say they showed that human serum may be oncolytic to human cancer cells In *1912 Kraus* showed that human serum may also lyse mouse cancer cells Since the advent of mouse ascitic tumours work on the oncolytic action of human serum has more or less been confined to its action on transplanted mouse ascitic tumours (see *Hartveit* 1963) As a result of this work all degrees of lytic ability have been ascribed to the serum from cancer patients Serum from non-cancer patients has also been shown on occasion to lack lytic ability, while the serum from normal individuals is said to be uniformly lytic

In view of this lack of conformity in different observers results the oncolytic activity of a series of sera from patients with different diseases and from normal individuals was investigated

MATERIAL AND METHODS

Sera Bl d was btain d fr m patients in the wards and fr m bl d d nors at Haukeland H spital Bergen by c urtesy f the medical and nursing staff Sera fr m 391 individuals were examined 56 from bl od donors 54 fr m cancer patients (h t l g ally verified) 141 from medical and 113 from surgical non cancer patients and 26 fr m pregnant women The bl od was all wed to cl t at r om temperature (0 C) and then spun gently The serum was decanted and ...
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The lytic action of human serum on cancer cells was first described by *Freund & Kaminer* in 1910 Their observation was that while the serum of normal individuals was able to lyse human cancer cells, the serum of cancer patients was often unable to do so, that is to say they showed that human serum may be oncolytic to human cancer cells In 1912 *Kraus* showed that human serum may also lyse mouse cancer cells Since the advent of mouse ascitic tumours, work on the oncolytic action of human serum has, more or less, been confined to its action on transplanted mouse ascitic tumours (see *Hartveit* 1965) As a result of this work all degrees of lytic ability have been ascribed to the serum from cancer patients Serum from non-cancer patients has also been shown, on occasion, to lack lytic ability, while the serum from normal individuals is said to be uniformly lytic

In view of this lack of conformity in different observers' results the oncolytic activity of a series of sera from patients with different diseases and from normal individuals was investigated

MATERIAL AND METHODS

Sera Blood was obtained from patients in the wards and from blood donors at Haukeland Hospital Bergen by courtesy of the medical and nursing staff Sera from 391 individuals were examined 56 from blood donors 54 from cancer patients (histologically verified) 141 from medical and 113 from surgical non cancer pa- tient [illegible]

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Experimental procedure Tumour cell suspensions of one vol whole tumour ascites in 20 vol physiological saline were made up One vol of tumour cell sus

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pension was mixed with one vol. of serum. In control preparations saline was used in place of serum.

Wet preparations were made up from these mixtures and incubated in a moist chamber at 37° C for 30 min., after which the preparations were examined microscopically for cell lysis, using a bright field condenser and cutting down the aperture.

The degree of lysis was recorded as follows:—

Non lytic	no lytic cells present
Lytic	Both lytic and non lytic cells present
Highly lytic	only lytic cells present

Two tests using cell suspensions from different mice were carried out on each specimen.

RESULTS

These are summarised in Table 1. All controls were negative, i.e. non lytic.

TABLE 1

The Oncolytic Ability of Fresh Human Serum from Normal Individuals (Blood Donors), Hospital Patients and Pregnant Women

Source of serum	No. of cases	Oncolytic ability of serum percentage			
		Non lytic	Lytic	Highly lytic	Total oncolytic
Blood donors	5[illegible]	77	[illegible]	14	23
Cancer patients	54	47	33	20	53
Medical patients	141	87	3	10	13
Surgical patients	113	60	24	16	40
Pregnant women	27	48	33	1[illegible]	5[illegible]
Total	391	70	16	14	30

see text

Of the total of 391 sera 70 per cent were non lytic, 16 per cent lytic and 14 per cent highly lytic. The repeat tests gave the same results. On further analysis differences between the lytic ability of sera from different groups of individuals were found. On combining the lytic and highly lytic sera into one group—oncolytic sera—in contrast to the non lytic sera, it was found that the cancer patients and pregnant women show the highest percentage of oncolytic sera (53 and 52 per cent respectively) with surgical patients (40 per cent) next, blood donors (23 per cent) and medical patients (13 per cent) last.

These variations were analysed further. I am indebted to the hospital staff for the access to clinical details.

Blood donors. No relationship was found between the lytic ability of the serum and the age, sex, *ABO or rhesus blood group of the donor.*

Cancer patients. No clear relationship to age or sex, or to the extent of the disease was found. More material is needed before it is possible to say if the variations are related to the type of cancer.

Medical and surgical non cancer patients. The marked difference in

the percentage of lytic sera in these two groups is not related to age or sex The only diagnosis that occurred frequently enough to allow any conclusion to be drawn was that of coronary artery disease There were 34 such cases 18 with recent symptoms of infarction All but one of these patients had non lytic serum Serum from the exception was lytic but not highly so Repeat specimens gave the same result The negative findings in this group can not however, in themselves explain the excess of non lytic sera in medical compared to surgical patients

Pregnant women The lytic ability of the serum was not related to the duration of the pregnancy

DISCUSSION

The present findings show that there are differences in the oncolytic ability of serum from different groups of patients The tests were carried out under controlled conditions (see *Hartveit* (A)) and the results proved to be reproducible The findings in the different groups can therefore be compared They show that serum from cancer patients may be either lytic or non lytic thus confirming both the previous positive and negative reports in the literature (*Bolande* 1960 *Kuru et al* 1959) Serum from pregnant women gave strangely similar results while medical non cancer patients—in particular patients with coronary artery disease—often showed non lytic serum These findings can not as yet be explained

The oncolytic activity of human serum on Ehrlich ascites carcinoma cells has been shown to be due to its complement content alone (*Hartveit* 1965 and (A)) so at first sight these results appear to suggest a lack of correlation between the total serum complement level and malignant disease On the other hand raised complement levels have been reported in cancer patients (*Fischel* 1953) and lowered levels in tumour bearing mice (*Hartveit* (C)) So on second thoughts it becomes evident that the situation may not be so simple

Recent work has shown that the Ehrlich ascites carcinoma cells are sensitized cells (*Hartveit* 1965 and (A)) The antibody in this case has been supplied by the host mouse in response to the antigenic stimulus of the homografted tumour It has also been shown that tumour cells may become sensitized in an otherwise genetically compatible mouse system *Hartveit* (B)) These cells Bergen A4 ascites carcinoma cells then behave in the same way as the Ehrlich ascites carcinoma cells—that is to say they lyse in the presence of fresh human serum and are capable of absorbing complement from such serum This latter tumour host system is comparable genetically to that of the cancer patient and his tumour Therefore the possibility that human cancer cells may also be sensitised cells must be considered *Wallheim & Ivy's* (1959) finding that human cancer cells may lyse in the presence of fresh human serum supports this view as does *Freund & Kaminer's* (1910) original observation

If cancer cells are sensitized they can be expected to absorb complement *in vivo*. This would reduce the complement content of the patient's serum. If this serum were then tested against Ehrlich ascites carcinoma cells it could be expected to be non-lytic but for one possibility. That is that the Ehrlich cells themselves could have absorbed complement as well as antibody *in vivo* (*Hartveit* (C)). The only complement component that is present in any appreciable amounts in the mouse is C1 (*Rice & Drowson* 1950, *McGhee* 1952), so the cells could supply C1 to the system. If then C1 were the factor lacking in the patient's serum—as it might well be as it is the first factor used up by sensitised cells (*Kabat & Meyer* 1961), this could be supplied by the mouse, with the result that the patient's serum would appear to be oncolytic. This point, that the mouse could supply C1 to the oncolytic system, may be of importance in view of the suggestion made by *Ginsberg et al.* (1961) that the C4 content of the sera of cancer patients may be reduced. Such sera should be non-lytic in this system.

The results in the blood donors show that normal human serum may have a wide range of oncolytic activity. This has not been stressed previously and is, at least, of practical importance. As there is such a wide range in the normal oncolytic activity of human serum, differences in disease will be difficult to interpret unless it can be shown, as suggested above, that there may be a difference not only between a positive and negative result, but also in the mechanism of the positive and negative results themselves.

SUMMARY

The lytic activity of 391 human sera was tested against Ehrlich ascites carcinoma cells. Both lytic and non-lytic sera were found in all groups examined, including blood donors. Cancer patients and pregnant women gave many highly lytic sera, while medical non-cancer patients—in particular those with coronary artery disease—gave few. It is suggested, on theoretical grounds, that it may be possible to analyse these differences further as the mechanism of the oncolytic reaction may differ in the different groups.

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The results in the blood donors show that normal human serum may have a wide range of oncolytic activity. This has not been stressed previously and is, at least, of practical importance. As there is such a wide range in the normal oncolytic activity of human serum, differences in disease will be difficult to interpret unless it can be shown, as suggested above, that there may be a difference not only between a positive and negative result, but also in the mechanism of the positive and negative results themselves.

SUMMARY

The lytic activity of 391 human sera was tested against Ehrlich ascites carcinoma cells. Both lytic and non-lytic sera were found in all groups examined, including blood donors. Cancer patients and pregnant women gave many highly lytic sera, while medical non-cancer patients —in particular those with coronary artery disease—gave few. It is suggested, on theoretical grounds, that it may be possible to analyse these differences further as the mechanism of the oncolytic reaction may differ in the different groups.

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Fig 1

Photom rograph of e le fr m Ca e 1 Haemat xyl n eo n Magn f cat on × 32

l ft and the r ght buc al mucosa
s ft palate cl se to the
adjacent a leucoplak a l
mu osa opp te the low
e ed and a b ops taken
All thre b p s were
and Mall r s a n ng
lengthened ul ep hel a

f an paque l n k

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The Department of Oral Pathology, Government Dental College
and Hospital Bombay,
and Department of Oral Pathology, Royal Dental College Copenhagen

FORMATION OF VESICLES IN ORAL SUBMUCOUS FIBROSIS

By

J J PINDBORG and BALDEV SINGH

Received 9 xi 64

Oral submucous fibrosis has always been considered a disease of the connective tissue and very little attention has been paid to changes in the oral epithelium A few authors (*Joshi* 1953, *DeSa* 1957, and *Sirsat & Khanolkar* 1962) described the occurrence of vesicles in the initial stages The vesicles are mainly located to the buccal mucosa and palate When the vesicles rupture, they turn into superficial ulcerations and heal by fibrosis *DeSa* states that cultures of the vesicular fluid fail to reveal any specific organism

So far, no reports are available on the histopathology of these vesicles During an investigation of a number of patients with submucous fibrosis the present authors were able to remove vesicles in toto from two patients and examine these two vesicles histologically

CASE REPORTS

Case 1—Six months before examination a 22 year old man had blisters on his
in the mouth and had difficulty in
in without tobacco At admission he
oft palate The mucosa in the palate
the cheeks and anterior pillars had a blanched appearance and marked fibrous bands could be felt One of the palatal vesicles was dissected under local anaesthesia

The vesicle was cut in serial sections and the histological examination revealed the presence of a subepithelial vesicle (Fig 1) In a limited area the epithelium is lifted up by an accumulation of fluid The epithelium covering the visicle is atrophic and unkeratinized The adjoining epithelium is slightly atrophic and the rete pegs have disappeared Lamina propria is the seat of a rather marked fibrosis In the juxtaepithelial zone there is a large number of thin walled vessels The inflammatory reaction in the connective tissue below the vesicle is minimal There are a few lymphocytes and polymorphonuclear leucocytes some of which are eosinophils

Case 2—A 40 year old man had had no complaints regarding his mouth when he was examined His submucous fibrosis was discovered incidentally The patient has taken pan with tobacco and smoked 10 bidis daily for 25 years The clinical examination revealed the presence of fibrous bands in the right buccal mucosa Both the

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Supported by a grant from the *Danish State Research Foundation*

Requests for reprints should be addressed to *J J Pindborg* 4 Universitetsparken Copenhagen Denmark

Case 1 was examined at the Dental College Lucknow The authors wish to thank Dr *T N Chawla* for cooperation

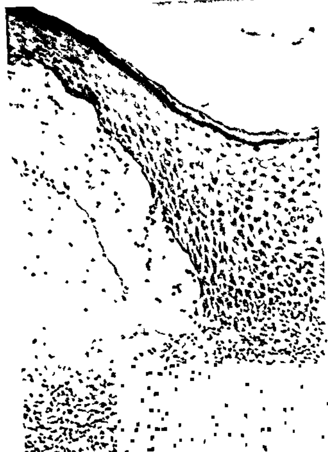


Fig 3

High power photomicrograph of area framed in Fig 2
Note the subepithelial nature of the vesicle Haematoxylin eosin
Magnification × 320

after *Sirsat & Khanolkar* 1960, demonstrated changes similar to human submucous fibrosis by painting rat palates with capsaicin, the active irritant principle of chillies

SUMMARY

Two patiens with oral submucous fibrosis are reported in whom vesicles were surgically removed The histological examination revealed in both the cases occurrence of *subepithelial vesicles* associated with eosinophilic cells in the connective tissue

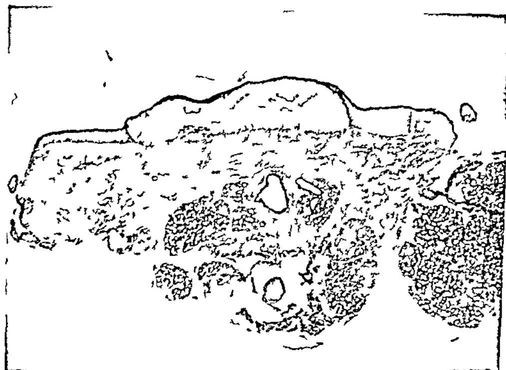


Fig 2
Low power photomicrograph of vesicle from Case 2 Haematoxylin eosin

DISCUSSION

In the two cases reported here the vesicles formed were undoubtedly subepithelial No remnants of the basal cell layer of the epithelium were found on the connective tissue surface under the vesicles Due to pressure from the underlying fluid the epithelium covering the vesicles was atrophic In Case 1 the inflammation was minimal whereas it was marked in Case 2 In both patients a number of eosinophilic cells were observed

Subepithelial vesicles or bullae of the oral mucosa are seen in the following conditions pemphigoid dermatitis herpetiformis Duhring epidermolysis bullosa hereditaria and bullous lichen planus In erythema multiforme exudativum vesicles are observed intra or subepithelially Occurrence of many eosinophils may also be noticed in some of these diseases

It would be tempting to assume an allergic reaction because of the eosinophilic cells in submucous fibrosis As the disease almost exclusively occurs in Indians a possible allergen should probably be found in the food *Sirsat* 1958 As a number of patients has never been taking tobacco or betel these habits are most likely not responsible for the development of submucous fibrosis The histological demonstration of subepithelial vesicles in submucous fibrosis should encourage further studies on a possible allergic nature of this condition and the more so

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The Department of Genetics, University of Hawaii, U.S.A.

ISOZYME STUDIES OF SOME HUMAN CELL LINES[1]

By

L. BECKMAN[2] and J. D. REGAN

Received 20.xi.64

The use of human cell cultures for the study of human genetic traits not amenable to the classical methods of human genetics has for the last decade been a source of many interesting studies. Human cell culture genetics has certain limitations, however, the most serious of these being (1) the complexity of even the most minimal cell culture medium, especially in that most cells require some amount of serum, and (2) the limited number of genetic markers available.

Identification of the enzymes present in cultured cells, and in particular the discrimination of multiple molecular forms of an enzyme, isozymes (*Markert & Møller* 1959), by starch gel electrophoresis (*Smithies* 1959) makes available a class of genetic markers which have had relatively little use before.

Maio & DeCarli (1962) found that sub-clones of the HUF strain differed considerably in their alkaline phosphatase activity. In some clones with low enzyme activity the alkaline phosphatase could be induced by cultivation in the presence of either prednisolone or phenyl phosphate. Preliminary results also indicated that in one sub-clone there were at least three electrophoretically different alkaline phosphatase zones.

Paul & Fottrell (1961) described the esterase pattern in uncultured human cells, in cultured human cells from skin, kidney and heart and in HeLa cells. In all the cultured human cells there was a typical esterase pattern which was distinct from the pattern found in cultured mouse cells. There was essentially no difference between the ester...

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[2] Contribution no. 31 of the Pacific Biomedical Research Center.

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De Carli et al (1963) found a correlation between alkaline phosphatase activity and the presence of small acrocentric chromosomes The finding of increased leucocyte alkaline phosphatase in Down's Syndrome patients (*Lennox et al* 1962) is of interest in this regard

This is a report of isozyme studies on some continuous human cell lines and primary cells

MATERIALS AND METHODS

The cell lines studied were WISH amnion (*Hayflick* 1962), HeLa (*Gey et al* 1952), and RA a new line of human amnion (*Regan & Loh* data to be published) The WI 38 semi continuous cell strain (*Hayflick & Moorhead* 1961) was also examined For comparison to primary tissue homogenates from three fresh amnions and from amnion epithelial cells cultivated for ten days *in vitro* were investigated as was human and calf serum The cells were grown in 32 oz prescription bottles under standard conditions on Eagle s Basal Medium with 10 per cent inactivated calf serum

Besides the control RA culture a number of RA cultures and in one case HeLa cells were grown in the presence of 10 µg/ml cortisone (11 dehydro 17 hydroxicorticosterone monoacetate) to test its effect on the zymogram patterns

The cells were harvested by scraping them from the glass washed twice in saline and homogenized in a minimal amount of fluid by a combination of mechanical treatment and freeze thawing The homogenates were absorbed into small pieces of filter paper and subjected to starch gel electrophoresis For the esterase and alkaline phosphatase zymograms the discontinuous buffer system by *Poulik* (1957) was used while for the studies of leucine aminopeptidase and catalase another discontinuous Tris buffersystem was found to be more suitable (*Ashton & Braden* 1961) For the staining of esterases α naphthyl acetate was used with Blue RR salt as dye coupler Alkaline phosphatase was stained with α naphthyl phosphate and Blue RR salt Leucine aminopeptidase (LAP) was determined by the use of 1 leucyl α naphthyl

of the peroxide except in the areas where the peroxide had been destroyed by the catalase (cf *Paul & Fottrell* 1961)

RESULTS

Staining of the starch gels with Amido Black did no reveal any clearly defined protein zones in the homogenates from the cell lines The samples rather appeared as diffuse columns clearly delineated from one another by the non staining space between them The esterase zymograms revealed a pattern that agreed very well with the previous descriptions (*Paul & Fottrell* 1961 *Komma* 1963) There is a series of slow moving zones and one or two fast moving zones The fast zones had a mobility close to that of the albumin associated esterase found in human serum (see Fig 1) Essentially the same general pattern was found in primary amnion the RA line the WISH amnion line the WI-38 strain, and the HeLa cells Human serum has a quite different pattern There were some minor variations between homogenates from different cultures Thus in some harvests from the RA line there was only one fast zone instead of the usual two We have also seen two cultures of RA cells with practically no trace of the series of slow bands but with two very strong fast bands

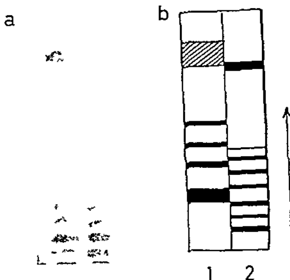


Fig 1

Esterase zymogram pattern in the RA amnion line a) photograph of starch gel, b) schematic picture showing human serum esterases (1) compared to the esterases of the RA cells (2) The arrow shows the direction of migration towards the anode

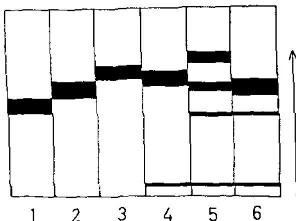


Fig 2

Schematic picture showing the alkaline phosphatase patterns in three different primary amnions (1 3) human serum (4) normal RA cells (5) and RA cells grown in the presence of cortisone (6) The four bands seen in sample 5 are called A B C and D in decreasing order of mobility towards the anode

Multiple alkaline phosphatase bands were observed in all three continuous cell lines studied (see Figs 2 and 3) The calf serum used in the medium did not show any alkaline phosphatase zone, nor did the WI 38 strain and the primary amnion epithelium cultivated for 10 days *in vitro* Extracts from fresh amnion showed only one enzyme band

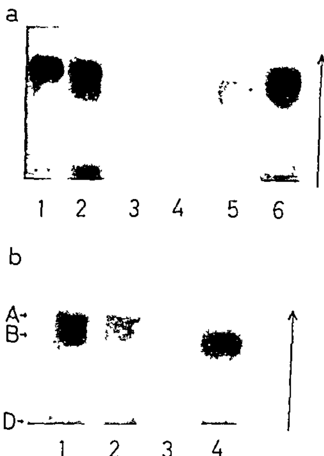


Fig 3

Photographs of starch gels showing alkaline phosphatase variations a) HeLa cells (1) WISH amnion (2) two RA cultures showing weak alkaline phosphatase (3 and 4) human serum (5) and RA cells with strong fast band (6) b) Variations in the RA line Cells derived from survivors of 800 r (1) untreated culture (2) a sub cloning of RA (3) and cortisone treated cells (4) Samples 1 and 2 show the A B and D zones 3 only the A zone and sample 4 has the B and D zones

which stained strongly Three different amnions were studied and each of them had a distinct electrophoretic variant of alkaline phosphatase (Fig 2, samples 1–3) In the HeLa, WISH and RA lines we observed two distinct fast-moving zones (corresponding to zones A and B in Fig 2) and one slow-moving zone close to the start (zone D) Zone A of the cell lines was faster than the alkaline phosphatases of fresh human amnion and serum The alkaline phospatases of the RA line were followed continuously for six months in a series of 18 harvests of cultures During the first month zone A was predominant, then for a period zone B became more pronounced and zone A weaker and in the last month of study zone A was stronger again and zone C more clearly visible than previously A few RA cultures gave weak enzyme zones (Fig 3a, samples 3 and 4

In 21 different cultures of RA cells grown in the presence of cortisone (10 μg/ml) and sampled over the whole period of study, the alkaline

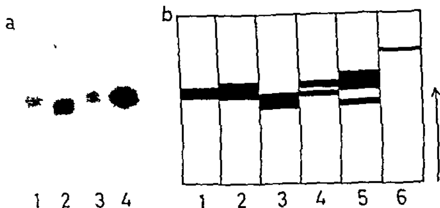


Fig 4

Variations in leucine aminopeptidase a) photograph showing calf serum (1), primary amnion (2) RA cells (3) and human serum (4) The extra fast moving zone in samples 1 and 4 is due to unspecific staining of serum albumin b) schematic picture showing calf serum (1) human serum (2) primary anion (3) RA cells (4), WI-38 strain (5) and faint LAP band sometimes seen in RA and HeLa cells (6)

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Catalase activity was found in homogenates from all cultured cells and in primary amnion but not in calf serum The mobility of the single catalase zone was rather variable Cultures with slow or fast enzyme bands were found both in the RA line and the HeLa cells It is not possible to ascribe any *specific pattern to a certain cell line or tissue*

DISCUSSION

Four different enzyme systems were detectable in human cells grown *in vitro* It is of importance to clarify to what extent the enzyme activity found in the homogenate can be influenced by the addition of protein from the calf serum used in the medium In the case of alkaline phosphatase and catalase no enzyme activity was found in calf serum and

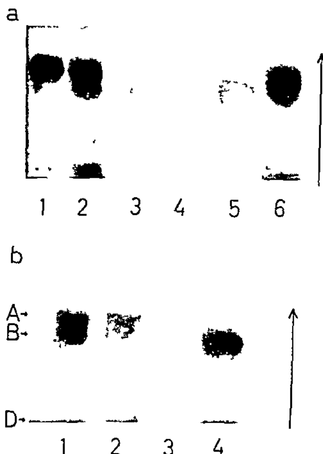


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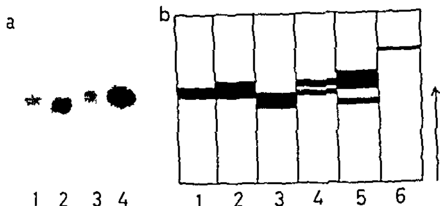


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the esterase and the leucine aminopeptidase enzymes were different from the ones found in serum Hence, artefacts originating from the proteins of the medium can be excluded The esterase patterns found were in good agreement with the findings by *Paul & Fottrell* (1961) and *Komma* (1963) There are no striking variations between primary tissue and cell lines grown for many years, which suggests a high degree of stability of the esterase marker On the other hand the fast esterase zones and the series of slow moving zones show strength variations, which, however, seem to be rather independent, *e g* , some cultures displayed very strong fast zones, while the series of slow esterase bands was staining weakly This suggests that those two groups of esterases may be controlled by genes located on different chromosomes and that the variations are due to dynamic changes in the chromosomal stemline

The strongly staining alkaline phosphatase enzymes in the primary amnions are probably derived from the deeper amnion layers (*Bourne* 1962) Trypsinization of amnion for primary cultures usually releases only the epithelial cells which show little or no alkaline phosphatase activity (cf also *Fortelius* 1963) It is possible that the alteration into a continuous cell line is accompanied by the emergence of a series of different molecular forms of alkaline phosphatase Of particular interest is the occurrence of a fast-moving zone in all three continuous cell lines, although the indistinguishable mobilities of the fast zones in the three lines is, of course, no proof of molecular identity A whole series of electrophoretically different alkaline phosphatase enzymes have been identified in human serum (*Boyer* 1961) Three of the enzyme zones (by *Boyer* called A, B and D) occurred only in the sera of pregnant women and it was demonstrated that these enzymes were synthesized in the placenta Another serum alkaline phosphatase zone has been found to be under the genetic control of at least three genetic loci two of which are the secretor and the ABO loci (*Beckman* 1964) Apparently a number of genes are controlling the human alkaline phosphatases and though essentially the same genetic information is present in every cell it is not always expressed Thus in the placenta some particular alkaline phosphatases are synthesized (induced) The culture conditions may have caused an induction in the continuous cell lines of enzymes that are not normally synthesized in epithelioid tissue The variability in the strength of the different alkaline phosphatases observed with time in these studies may possibly reflect dynamic changes in a heterogeneous cell population In the RA line cortisone can apparently interfere in some way with the synthesis of the fast-moving alkaline phosphatase zone A Zone B seems rather increased in cortisone treated cultures Studies of clonal derivatives of the RA line and of survivors of x-irradiation are in progress (see Fig 3b, samples 1 and 3) The RA clone seen in Fig 3b is interesting in that it shows only the zone A

The results concerning the LAP variations in the RA line suggest that this cell line may be inducible for LAP, while *e g* the WI-38 strain is

constitutive for LAP The mechanism of induction is not yet clarified Clear LAP zones have been found only in six cortisone treated cultures but most cortisone-treated cultures were negative Studies of the mechanism of LAP induction are under way

The electrophoretic variations of catalase were rather irregular and only a single blurred, wide zone was seen Thus catalase does not seem to be a very informative enzyme marker in studies of cells cultivated *in vitro*

SUMMARY

Electrophoretic variations in esterase, alkaline phosphatase leucine aminopeptidase and catalase were studied in some human cell lines (HeLa WISH amnion and RA amnion), the WI 38 strain, primary amnion and in serum

There is little variation between the esterase patterns in fresh tissue and in cells cultivated *in vitro* for many years suggesting a high degree of stability for the esterase enzymes The RA cells show four different alkaline phosphatase components They are apparently a rather delicate system subject to considerable variation due to dynamic fluctuations in the population of cells Cortisone is capable of interfering with the appearance of the fastest alkaline phosphatase zone when the cells are cultured in the presence of the hormone but not when the hormone is merely mixed with the homogenate Two other continuous cell lines studied (HeLa and WISH amnion) had a zone electrophoretically in distinguishable from the fastest alkaline phosphatase of RA The WI 38 cell strain and several samples of primary amnion both fresh and cultured briefly apparently did not possess this rapidly moving alkaline phosphatase

Leucine aminopeptidase activity appeared weak and variable in the cell lines studied but was strong in the WI 38 cell strain showing two enzyme zones

Catalase activity appeared as a wide blurred zone of variable electro phoretic mobility in the cell lines studied

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THE MUTAGENIC EFFECT OF HYDROXYLAMINE ON *ESCHERICHIA COLI*

By

SVERRE LIE

Received 25 iv 64

Hydroxylamine (HA) was first reported as a strong mutagenic agent by *Freese and coworkers* in 1961 (4) Working with phage T4, they found that the mutagenic effect was obtained only at high concentrations of HA and salt (NaCl) while low concentrations gave a rapid inactivation of the phage particles Under optimal conditions as much as 5 per cent of the phages could be induced to mutate in the r-region of the phage chromosome

HA attacks the base cytosine (1, 5, 8) and alters the structure of the base in such a way that it can pair with adenine instead of the usual guanine During DNA replication this change would result in a base pair transition (5)

HA has been found to be an effective mutagen for animal viruses (11) tobacco mosaic virus (9) and for transforming DNA (6) It does also induce chromosome breakage in mammalian cells (10)

It is the purpose of the present paper to describe the effect of HA on the mutation rates in *E coli* In this connection an investigation of the gross inactivating effect was deemed relevant

MATERIALS AND METHODS

Bacterial strains The test microbe used was the E coli K 12 strain T 71 which has previously been used in this laboratory (7)

Culture technique Heart infusion broth (HIB) and agar (both from Difco) were used as solid and liquid complete media Minimal medium was that
Davis & Mingioli (2)
plete or minimal liqu
the cold (+4° C) was
a titre of approximate

HA treatment The procedure was based on that described by *Freese et al*

Hydroxylamine mixture $NH_2OH \cdot HCl$ was dissolved in 2 × concentrated buffer Such solutions were never kept for more than one week Immediately before the addition of bacteria the pH was brought to 7.5 by means of concentrated NaOH

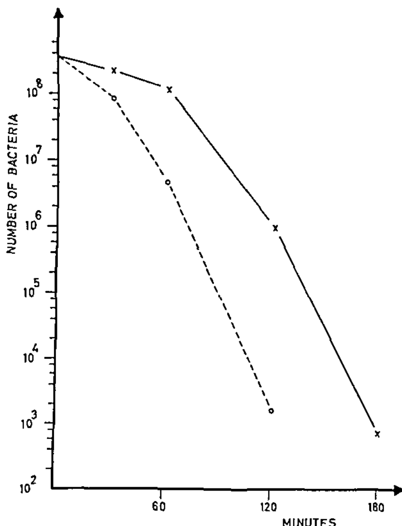


Fig 1
Inactivation curves of E coli grown in complete broth when exposed to 1 0 M Hydroxylamine (—x——x——x—) and 0 1 M Hydroxylamine (- - o - - - o - o - -) Salt concentration in both cases 1 35 M NaCl

(12 5 M) NaCl was added to the desired concentration and pH again adjusted to 7 5 At zero time the bacterial suspension was added and incubation started in a 37° C waterbath

Genetic methods The inactivating effect of HA was analysed by determining the decrease in viable cell count employing a plate dilution method In each determination the titre obtained represented the mean of the number of bacteria on at least three plates Mutagenic effects were determined by an estimation of mutations from streptomycin sensitivity (str s) to streptomycin resistance (str r) and from dependence to non dependence of a particular growth factor Details concerning these techniques have been described in the result section

RESULTS

The Inactivating Effect of Hydroxylamine

In phage the inactivating effect of HA is greatest at low concentrations of the compound (4) From the experiment presented in Fig 1 it is seen that the same is also true for cultures of *E coli* in the stationary

growth phase When the cells are exposed to 0 1 M HA the inactivating effect is significantly greater than when exposed to 1 M of the compound

In the two curves presented the salt concentration is the same (1 35 M NaCl) This is taken to indicate that the concentration of HA is the main factor determining the rate of killing When the salt concentration was decreased to 0 2 M NaCl, inactivation curves were obtained which were identical to those presented in Fig 1 Thus, in contrast to the situation in phage (4) the salt concentration seems to be of little or no importance in determining the rate of killing in *E coli*

Successive series of experiments showed that concentrations of HA below 0 03 M were without significant inactivating effect in the technique used On the other hand, concentrations above 3 M gave non-specific killing, presumably due to the high osmotic pressure of the reaction mixture

In the experiments reported in Fig 1, the cells were grown in complete media (HIB) Growth in minimal media regularly resulted in cells with greater resistance towards HA With such cells the inactivation curves from separate experiments did not correspond so well The highest concentration of HA, however, always gave the slowest inactivation rate

The Mutagenic Effect of Hydroxylamine

The mutagenic effect of HA on cells of *E coli* was first studied in a system which employed the mutation from streptomycin sensitivity to streptomycin resistance In the experiments presented in Table 1, cells were incubated in a reaction mixture which contained 1 2 M HA and 0 8 M NaCl The zero time control consisted of cells and salt solution (1 5 M NaCl) only After an exposure time such as indicated the cells were diluted 1 10 in stopping mixture, centrifuged and resuspended in saline to the original volume before plating on complete agar plates After various hours of incubation, the agar was transferred to another agar plate of the same composition and volume which in addition contained 200 μg streptomycin per ml After another 48 hours of incubation resistant colonies were counted

TABLE 1

The Mutagenic Effect of Hydroxylamine (HA) on E coli *Number of Streptomycin Resistant Mutants per 10⁹ Bacteria*

Incubation hours	Time of exposure to HA (min)		
	0	90	180
0	0	0	0
3	3 2	146	1786
5	*	263	2285

Reaction mixture 1 2 M HA 0 8 M NaCl The numbers represent the mean of three plates

* Too heavy background growth of sensitive cells before exposure to streptomycin

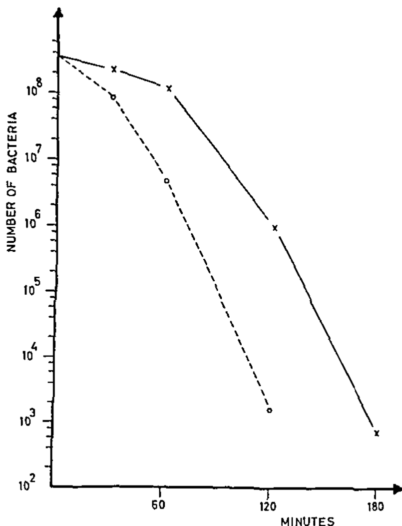


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phenomenon was first reported in phage (4) and has been found also in animal viruses (11) This paper shows that the same is true for bacteria One explanation proposed (4) is that in a concentrated solution of HA there will be inactive ions present (*e g* NH_3OH^+). Such ions may attach to the sensitive sites of the cell making these resistant to the action of HA

The salt concentration of the reaction mixture does not influence the rate of bacterial killing, a phenomenon which is in contrast to the situation in phage This could indicate that the main target of HA in bacteria is intracellularly located in a milieu of salt concentrations which are hardly influenced by that of the environment

The inactivation rate increases with time of exposure to HA Again this is analogous to the situation in phage (4) Purified RNA from tobacco mosaic virus, however, presents an exponential inactivation curve when exposed to HA (9) This would suggest that the alteration of a single base pair is sufficient for an inactivation of the whole molecule The more complex inactivation curves obtained with phage and bacteria could therefore suggest that besides an inactivation due to reactions with DNA, HA may inactivate by reacting with some other cell constituents, most likely proteins

HA exerts a great mutagenic effect on *E coli* Mutations are induced both from str s to str-r, and from dependence to non-dependence of arginine in an arginine-requiring mutant In both cases a period of active metabolism and replication is needed for the induced mutations to become expressed The model of HA action would predict this, since the altered DNA-molecule must pass through two or more replications in order to get the base-transitions introduced If great numbers of auxotrophic mutants were studied, only those which originally mutated by a transition from $\frac{A}{T}$ to $\frac{G}{C}$ should be found to revert upon treatment with HA

Only one report has been found in which the mutagenic effect of HA on bacteria was examined (3) *Eisenstark & Rosner* studied the reversion pattern of 200 mutants in the cysC region of Salmonella typhimurium LT 2 using a series of different chemical mutagens With HA no consistent positive results were obtained and the authors postulate that the substance is metabolized before it reaches the bacterial genome The method used, however, is only briefly described, thus the cause of the discrepancy between their results and the positive mutagenic effect obtained in this paper remains uncertain

SUMMARY

The inactivating and mutagenic effect of hydroxylamine (HA) on *E coli* have been studied The inactivation curves were complex with increasing inactivation rates with increasing time of exposure to HA

The data presented in Table 1 show that HA does have a strong mutagenic effect on *E. coli*. It is also evident that this effect depends upon the length of exposure to the compound. It might be argued that the increased fraction of resistant cells is due to some kind of selective killing of sensitive cells. This cannot be the case, however, since the absolute number of str-r colonies after 90 minutes exposure to HA is far greater than the number in the untreated population.

When the concentration of HA is decreased to 0.1 M a definite mutagenic effect is still observed on this particular mutation in *E. coli*. In these experiments, however, the effect is more difficult to detect due to the more rapid killing which excludes an exposure time of more than 60 minutes. Variation of the salt concentration from 0.2 M to 1.3 M NaCl did not influence the mutagenic effects observed.

TABLE 2

The Mutagenic Effect of Hydroxylamine (HA) on E. coli. Mutation from Dependence to Non-Dependence of Arginine (arg)

Time of exposure to HA (min.)	Number of arg^+ cells per 10^9 arg^- cells when plated on	
	M	M + HIB 1%
0	0	13
30	18	160
90	56	423

Reaction mixture: 1.0 M HA, 1.3 M NaCl. The numbers represent the mean of three plates.

In another series of experiments the mutation from dependence to non-dependence of a particular growth factor was studied. In the experiments presented in Table 2 an arginine-requiring mutant of the test microbe T 71 was grown in minimal medium supplemented with arginine for 25 hours. After treatment with HA the cells were plated on minimal media and on minimal media supplemented with 1 per cent broth (HIB). The number of residual divisions on this concentration of broth was determined by washing off the bacteria with saline at various time intervals and measuring the increase in viable cell count. With 1 per cent broth the number of residual divisions was found to be around five.

The data in Table 2 show that this strain of *E. coli* can be induced to revert to prototrophy upon treatment with HA. It is also evident that a great majority of the induced mutations needs replication or active metabolism in order to become expressed.

DISCUSSION

One of the striking features of the inactivating effect of HA is the dependence of the inactivation rate on the concentration of HA. This

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The Division of Pediatric Pathology at the Department of Pathology,
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PULMONARY HYALINE MEMBRANES OF THE NEWBORN

The Structure of the Membranes at Varying Postnatal Age

By

BENGT ROBERTSON[1]

Received 29 iv 64

The relationship between hyaline membranes of the newborn and other pulmonary lesions, particularly intra-alveolar oedema, has been discussed by several authors (6–8, 10–13, 15, 17, 18). It has been maintained that the membranes are probably formed by a process of "condensation" of oedema fluid (12, 15, 17, 18). The presence of fibrin in the hyaline membranes is well consistent with this view (2, 5).

The appearance of the membranes has been found to vary with the postnatal age of the patient. It is generally agreed that the thickness of the membranes increases with the survival time (10, 13) though the inverse relationship has also been reported (9). A "macrophage response" has been described in cases surviving the first few days (4, 18). Proliferative and reparative phenomena in the alveolar walls proper have also been recognized in these cases (1). The purpose of this study was to examine the morphological features of pulmonary hyaline membranes of the newborn at varying postnatal age. The results have in part been presented in previous papers (14, 15).

MATERIAL AND METHODS

The study was performed on 117 neonatal autopsy subjects, all of which had pulmonary hyaline membranes. The age of the patients varied between 6 hrs and 6 days. All cases had displayed clinical symptoms from the first day of life in the form either of respiratory distress or of episodes of cyanosis, multiple episodes of respiratory arrest or convulsions.

The term hyaline membrane is applied to the acidophil hyaline substance which lines the alveoli and/or alveolar ducts in a bandlike fashion. The [illegible] of the hyaline [illegible] The degree of [illegible] alveolar haer [illegible] denotes here [illegible] the peripheral air-spaces (alveoli, alveolar ducts). For details concerning the grading system in this study we refer to the original paper published elsewhere (15). The presence of cellular infiltration in the membranes and the alveolar walls proper or proliferation of the alveolar epithelium was also recorded, however, without the application of a fixed grading system.

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[1] Supported by grants from *Karolinska Institutets reservationsanslag* and *Statens Medicinska Forskningsråd*, *Föreningen Solstickan T 231*.

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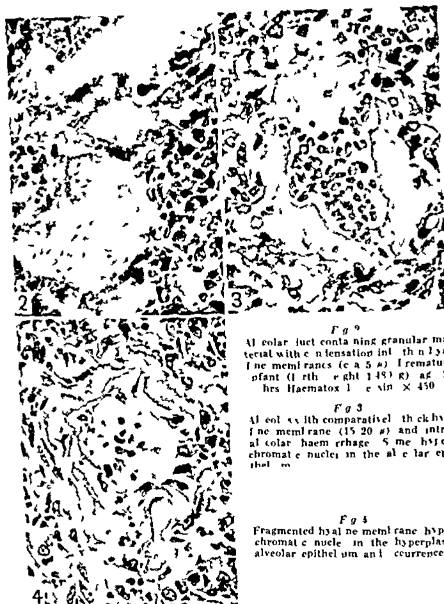


Fig 2
Al eolar luct conta ning granular ma terial with c n lensation inl th n hya l ne meml ranes (c a 5 µ) Premature nfant (l rth e ght 1 48) g) ag 21 hrs Haematox l e sin × 450

Fig 3
Al eol s ith comparativel th ck hya l ne meml rane (15 20 µ) and intra al eolar haem rrhage S me hyper chromat c nucle in the al e lar ep thel m

Fig 4
Fragmented hyal ne meml rane hyp r chromat c nucle in the hyperplasti alveolar epithel um an l ccurrence of

Cases exp r ng during the third and fourth day displayed thick mem l ranes (20 25 µ) whereas *intra* alveolar oedema was still less prom ment than in the second group Intra alveolar haemorrhage and inflam mation w s a rather *common finding* The alveolar ep thelium beneath the meml ranes was often hyperplastic with hyperchromatic nuclei The l orderline l etwe n membranes an l ep thelium was in places indistinct (Fig 4) Particularly *in the* older ones of these cases the membranes

Histologic specimens from the lungs were examined in the following stains haematoxylin eosin van Gieson and Lendrum's reticulum stain

RESULTS

The majority of the patients were premature and most of the infants, particularly the prematures, expired within the first 48 hours (*Fig 1*)

In cases dying within the first 24 hours, the hyaline membranes were generally thin (5–10 μ), and intra-alveolar oedema was a prominent finding Histologic pictures suggesting condensation of intra-alveolar oedema into thin hyaline membranes were often encountered in this age group (Fig 2) Inflammation or intra-alveolar haemorrhage were seldom present, nor was there any cellular infiltration in the hyaline membranes or the alveolar walls

In patients who expired during the second day, the membranes were generally considerably thicker (15–20 μ) and intra-alveolar oedema was not so prominent as in the former group Still no cellular infiltration in the alveolar walls or the membranes was encountered, but in some places there were hyperchromatic nuclei in the alveolar epithelium The borderline between membranes and alveolar walls was rather distinct (Fig 3)

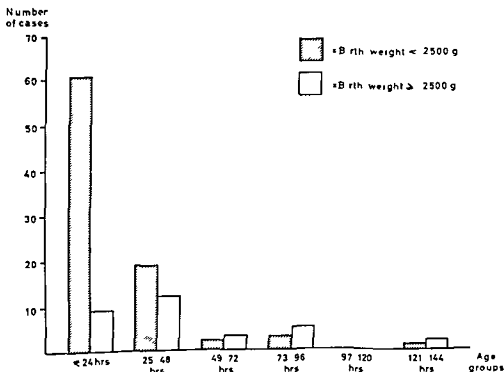


Fig 1

Distribution of the series (117 neonatal cases of pulmonary hyaline membranes 68 males 49 females) with respect to birth weight and age

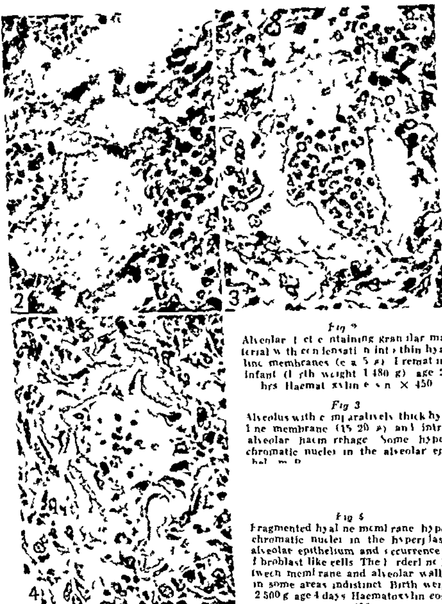
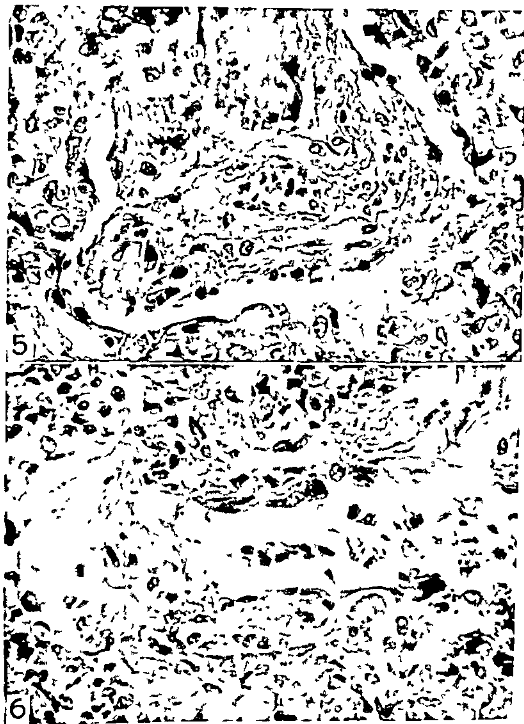


Fig 2
Alveolar pocket containing granular material with condensation into thin hyaline membranes (c a 5 μ) Premature infant (birth weight 1 480 g) age 21 hrs Haematoxylin eosin × 450

Fig 3
Alveolus with comparatively thick hyaline membrane (15 20 μ) and intra alveolar haemorrhage Some hyperchromatic nuclei in the alveolar epithelium

Fig 4
Fragmented hyaline membrane hyperchromatic nuclei in the hyperplastic alveolar epithelium and occurrence of fibroblast like cells The borderline between membrane and alveolar wall is in some areas indistinct Birth weight 2 500 g age 4 days Haematoxylin eosin × 450

Cases expiring during the third and fourth day displayed thick membranes (20 25 μ) whereas intra alveolar oedema was still less prominent than in the second group Intra alveolar haemorrhage and inflammation was a rather common finding The alveolar epithelium beneath the membranes was often hyperplastic with hyperchromatic *nuclei The* borderline between membranes and epithelium was in places indistinct (Fig 4) Particularly in the older *ones of these* cases the membranes



Figs 5 6

Fig 5 Fragmented hyaline membrane infiltrated by histiocytes The membrane is detached from the alveolar wall possibly by artefact Birth weight 2 840 g age 4 days Haematoxylin eosin × 530

Fig 6 Alveolar duct with hyaline membrane infiltrated by histiocytes The membrane merges with the wall of the duct Birth weight 4 800 g (diabetic mother) age 6 days Haematoxylin eosin × 420

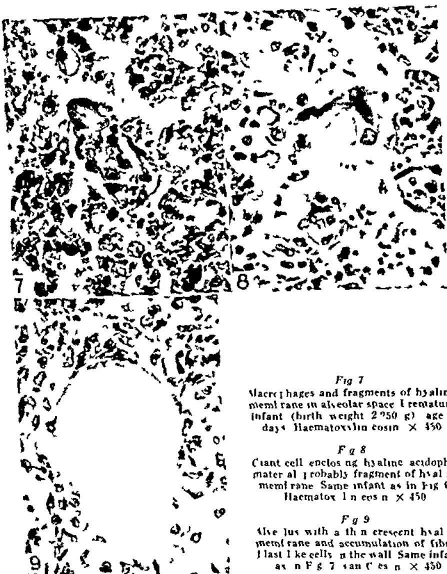


Fig 7

Macrophages and fragments of hyaline membrane in alveolar space Premature infant (birth weight 2 ?50 g) age 6 days Haematoxylin eosin × 450

Fig 8

Giant cell enclosing hyaline acidophil material probably fragment of hyaline membrane Same infant as in Fig 6 Haematoxylin eosin × 450

Fig 9

Alveolus with a thin crescent hyaline membrane and accumulation of fibroblast like cells in the wall Same infant as in Fig 7 [illegible] eosin × 450

were fragmented and infiltrated by histiocytes (Fig 5) In places the alveolar walls proper were thickened and showed excess of histiocytes and fibroblast like cells (Fig 4) No excess of collagen or ret[illegible]

[illegible] patients displayed thick hyaline membranes ([illegible]0 [illegible] μ) and practically no intra alveolar oedema *In the third* case the membranes were *thinner (10 15 μ)* and there was moderate intra alveolar oedema In all these cases there was some degree of in

flammation and intra alveolar haemorrhage The hyaline membranes were largely detached from the alveolar walls and fragmented, but in places the membranes seemed to be partly incorporated in the alveolar walls (Figs 6 7) The hyaline material was infiltrated by many histiocytes and some giant-cells were present in the alveolar spaces These giant-cells contained clumps of acidophil hyaline material, probably phagocytized fragments of hyaline membranes (Fig 8) As in the former age groups, the alveolar walls were often thickened and contained many histiocytes and fibroblast-like cells There was no excess of collagen or reticulin fibers in the alveolar walls (Fig 9)

COMMENT

The fact that the hyaline membranes tend to increase in thickness inversely with the degree of intra-alveolar oedema, suggests that the oedematous fluid "condenses" to form membranes, as has been pointed out earlier (12, 15, 17, 18)

The fragmentation of the membranes and the infiltration of these as well as of the alveolar walls by histiocytes and fibroblasts indicate a process of repair initiated on the third or fourth day of the disease These observations, which agree with those of some other workers (4, 18), have been interpreted as evidence of a previous damage to the alveolar walls It has been postulated that such damage probably is connected with the development of the hyaline membranes (1) Studies by the electron microscope have revealed destruction of the alveolar lining and the capillary walls in an early stage of the disease (2, 3) The cause of this destruction remains obscure but hypoxia may be a factor of importance The hyaline membranes per se seem to be removed at least in part by macrophages and it is probable that many of these cells are derived from proliferating alveolar epithelium The borderline between membranes and alveolar walls was often indistinct in cases surviving for more than three days This feature suggests that the membranes, during the process of repair, to some extent become incorporated into the alveolar walls

Similar features, *i e* hyperplasia of the alveolar epithelium, thickening of the alveolar walls with accumulation of histiocytes and fibroblasts, have been observed in patients with clinical histories suggesting hyaline membranes in the neonatal period, but dying at the age of 2 3 weeks of complications unrelated to their initial pulmonary disease (16) In these cases excess of collagen and reticulin fibers has been demonstrated in the thickened alveolar walls These features have been interpreted as a later stage of such process of repair as described above

Whatever the cause of this pulmonary disorder, it is evident that the hyaline membranes, when formed, initiate a "macrophage response" accompanied by reparative phenomena in the alveolar walls proper,

and it is probable that this process at least in some of the surviving cases may result in fibrosis of alveolar walls

SUMMARY

The morphologic appearance of pulmonary hyaline membranes was studied in 177 neonatal autopsy cases, varying in age between 6 hrs and 6 days The thickness of the membranes was found to increase with the survival time inversely with the degree of intra-alveolar oedema, which was a prominent finding in the cases dying at early stages In cases surviving for three days or more, the membranes were found to be fragmented and infiltrated by histiocytes and giant-cells Particularly in the six day-old cases the borderline between membranes and alveolar walls was often indistinct and the hyaline material seemed to some extent to be incorporated into the alveolar walls, which were thickened and infiltrated by histiocytes and fibroblast like cells It is suggested that these features, which indicate a process of repair, may result in fibrosis of alveolar walls, as has been observed in patients with clinical evidence of hyaline membranes in the neonatal period but expiring later, at the age of 2–3 weeks

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Acta path et microbiol scandinav 62 589 594 1964

The State Serum Institute, Helsinki and the Minerva Foundation Institute for Medical Research Helsinki Finland

OBSERVATIONS ON HUMAN COMPLETE AND INCOMPLETE 7S γ-GLOBULIN ANTIBODIES AGAINST RED CELLS AND THEIR INTERACTIONS WITH THE RHEUMATOID FACTOR

By

K Aho, J Leikola and K Simons

Received 29 x 64

We have previously studied the reaction of the rheumatoid factor (RF) with sheep erythrocytes sensitized with different subagglutinating doses of human 7S γ-globulin antibodies against sheep red cells, and with O Rh positive cells sensitized with incomplete anti-Rh antibodies (*Aho, Harboe & Leikola* 1964) The pattern of reaction of rheumatoid sera with sheep cells heavily sensitized with human anti-sheep cell antibodies and with O Rh positive cells sensitized with the rare "diagnostic" anti-Rh sera showed some basic similarities Correspondingly, the system using sheep cells lightly sensitized with human anti-sheep cell antibodies and that using O Rh positive cells sensitized with anti-Rh sera suitable for Gm typing resembled each other The purpose of the present work was to obtain additional information concerning this question by quantitating the γ-globulin fixed onto the cell surface under various experimental conditions

MATERIAL AND METHODS

Immune sera The production of the human anti sheep cell antibody sera has been described elsewhere (*Aho Harboe & Leikola* 1964) The immune anti AB sera were

... were used The rabbit anti human γ globulin serum No 139 (*Aho & Wager* 1961) was used in the anti globulin consumption tests

Fractionation of γ globulin[1] The serum to be fractionated was first dialyzed against 0 02 M phosphate buffer pH 8 0 whereafter it was transferred to a column

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The authors are highly indebted to Dr *M Harboe* of Oslo for his comments concerning the preparation of the manuscript and for valuable sera

[1] In the present work the three major classes of serum immunoglobulins are termed β A globulin β_{2} macroglobulin (β M) and 7S γ globulin or briefly γ globulin

of DEAE cellulose equilibrated with the same buffer. Elution with this same buffer gave a pure γ-globulin peak as shown by agar electrophoresis and immunoelectrophoresis. The effluent was concentrated by ultrafiltration. The γ-globulin fractions were stored at —20° C.

Anti-globulin consumption test. The test was performed by the dilution/absorption technique (*Steffen* 1963). Appropriate amounts of cell suspensions usually corresponding to 0.1 ml of packed cells were incubated with the antisera for 90 minutes at 37° C. When different cells were used in the same experiment the cell concentrations were adjusted by their haemoglobin content. Human albumin solution was usually added to the γ-globulin fractions of antisera to prevent non-specific aggregation of γ-globulin onto the cell surface. The sensitized cells were washed four times with a large volume of chilled phosphate buffered saline pH 7.4 and suspended in a 2 ml volume. From this six 0.25 ml aliquots were taken and mixed with equal volumes of the last six twofold dilutions of anti-γ-globulin serum preceding the final titre. After 15 minutes at 37° C, the cells were centrifuged and the supernates were separated and tested for the remaining anti-γ-globulin activity by a microscopic technique (*Harboe & Lundevall* 1959). As a detector system heavily sensitized Rh positive cells were used. The consumption results were recorded in half dilution steps based on the strength of agglutination in the last reactive dilution. The basic consumption of the non-sensitized cells was usually half a titre step.

RESULTS

Experiments with anti-Rh sera. Consumption experiments were performed with two diagnostic anti-Rh sera (anti-CD Ripley and anti-D Wiklinska) and with six other anti-Rh sera, three of which were suitable for the demonstration of anti-Gm(a). O Rh positive cells were sensitized with increasing amounts of each of the eight anti-Rh sera. The maximal consumption varied very little from serum to serum, although the amount of anti-Rh serum required for this maximal consumption displayed great differences. Four of these sera were titrated simultaneously and the results are shown in Table 1. It appears that the same amount of γ-globulin was fixed onto the cell surface from the "diagnostic" and from the other anti-Rh sera. Control experiments with the 7S γ-globulin fraction of one anti-Rh serum showed that the maximal consumption in this instance was not greater than that from the original anti-Rh serum.

TABLE 1

Consumption of Anti-γ-Globulin Serum by Anti-Rh Antibodies

Volume in ml of anti-Rh serum used for sensitizing 0.1 ml of packed ccDe cells	Anti-γ-globulin consumption titre			
	Anti-CD ripley	Anti-D wiklinska	Anti-CD liljemark	Anti-D halttu
0.8			3	3
0.4		3	2½	3
0.2	3	3	2	2½
0.1	3	2½	1½	1½
0.05	2½	2	1	1
0.025	2½	1	½	1
0.012	1½	1		
0.006	1			

not done

Experiments with human 7S γ globulin antibodies against sheep red cells Sheep erythrocytes were sensitized with increasing amounts of 7S γ globulin fractions from four persons (T P, P V, K A, and J L) and the anti globulin consumption titres were determined as above The results with a simultaneous comparison with two anti Rh sera, are shown in Table 2 It is seen that heavily sensitized sheep erythrocytes consumed markedly more of anti γ globulin serum than O Rh positive cells sensitized with an excess of anti Rh serum The sensitizing dose corresponding to this maximal uptake in the Rh system was 1/16 1/32 of the minimum agglutinating dose (MAD)

TABLE 2

Consumption of Anti γ Globulin Serum by Human 7S γ Globulin Antibodies against Sheep Red Cells

Cells sensitized with	Anti globulin consumption titre			
	T P	P V	K A	J L
1/2 MAD	6	6	6	6
1/4 MAD	5	6	5	5
1/8 MAD	4	5	4½	4
1/16 MAD	3½	4	3½	3½
1/32 MAD	2½	3	2½	2½
1/64 MAD	1½	2	2	1½

The maximal consumption of anti Rh sera *Liljemark* and *Hallila* (CcDe cells) was 3½ and 3 titre steps respectively
MAD Minimum agglutinating dose

TABLE 3

Consumption of Anti γ Globulin Serum by Human 7S γ Globulin Antibodies against Group AB Cells

Cells sensitized with	Anti globulin consumption titre	
	K S	L H
1/2 MAD	5	4
1/4 MAD	3½	3
1/8 MAD	2½	2
1/16 MAD	2	1½

The maximal consumption of anti Rh sera *Liljemark* and *Korpela* (CcDe cells) was 3 and 2 ½ titre steps respectively
MAD Minimum agglutinating dose

Experiments with human 7S γ globulin antibodies against AB cells The ability of the 7S γ globulin antibodies of K S and L H to render human group AB cells agglutinable by rheumatoid sera was at first studied Agglutination experiments with selected rheumatoid and normal sera revealed that the reaction pattern closely resembled that previously seen when human 7S γ globulin antibodies against sheep red cells were used as sensitizing agents The only difference observed was

that the anti-sheep cell system was slightly less dependent upon the sensitizing dose

Anti-globulin consumption experiments were then performed in a similar fashion as with the anti-sheep cell system The results are collected in Table 3 It appears that the heavily sensitized group AB cells consumed more of the anti-γ-globulin serum than the O Rh positive cells maximally sensitized with incomplete anti-Rh sera The sensitizing dose corresponding to this uptake was 1/4–1/8 MAD, *i e*, somewhat less than that in the anti-sheep cell system

DISCUSSION

Evidence has accumulated indicating that the incomplete anti-Rh antibodies are of the 7S γ globulin type, whereas the saline agglutinating antibodies are of the β_2-macroglobulin type (*e g*, *Fudenberg, Kunkel & Franklin* 1959) Recently, *Greenbury, Moore & Nunn* (1963) demonstrated that rabbit β_2M antibodies agglutinate red cells much more efficiently than 7S γ-globulin antibodies The rare human Derythrocytes also are agglutinated by the incomplete anti-Rh antibodies (*Race, Sanger & Selwyn* 1951), suggesting that these erythrocytes contain more D receptors than the other D positive erythrocytes It is possible that the agglutination is due to traces of saline agglutinating β_2M antibodies, although the observation gives a hint that the incompleteness of the 7S anti-Rh antibodies might be due to the limited number of available Rh receptors

In the present work, anti-globulin consumption experiments were performed with cells sensitized with some human complete and incomplete 7S γ-globulin antibodies The recent experiments of *Forsler* (1963) have shown that this test can be considered a useful semiquantitative technique for the demonstration of bound γ-globulin It was observed that sheep red cells and human group AB cells heavily sensitized with the 7S γ-globulin antibodies consumed more of the anti-γ-globulin serum than Rh positive cells sensitized with an excess of anti-Rh serum When the sheep cells or the AB cells were sensitized with an amount of γ-globulin corresponding to the maximal uptake in the anti Rh system, the cells were not agglutinated and the antibodies thus were incomplete in the same manner as the 7S anti Rh antibodies

Our previous results (*Aho, Harboe & Leikola* 1964) suggested that the reaction of the RF with sheep cells lightly sensitized with human anti-sheep cell antibodies is essentially an interaction of the RF with individual γ-globulin molecules whereas when heavily sensitized cells are used the reaction with immune aggregated γ-globulin plays a prominent rôle The present experiments further emphasize the significance of the amount of sensitizing γ-globulin fixed on the cell surface With heavily sensitized cells, the anti-sheep system and the anti AB system could be used "diagnostically" for the demonstration of the RF, and in these

instances the amount of γ-globulin fixed onto the cell surface was greater than in the anti-Rh system It seems reasonable to assume that the limited number of available Rh receptors does not usually provide conditions for intermolecular interactions of the antibody molecules, and consequently this system is not usually suitable for the demonstration of those RF components reacting with immune aggregated γ-globulin

The few 'diagnostic' anti Rh sera make an exception Our previous results (*Aho, Harboe & Leikola* 1964) indicate that the Gm type is not crucial in determining whether an antiserum can be used "diagnostically", and it appears in the present work that the cells are not more heavily coated in the "diagnostic" than in the usual anti-Rh systems Thus their unusual serologic behavior remains difficult to explain, although it may be speculated that the antibody molecules in these sera are more liable to configurational changes characteristic of the immune aggregated state than in other anti-Rh sera

SUMMARY

Anti globulin consumption experiments were performed with cells sensitized with some incomplete and complete 7S γ-globulin antibodies In the anti Rh system the consumption varied very little from serum to serum if an axcess of anti Rh serum was used for sensitizing the cells No difference was observed between the "diagnostic" and the other anti Rh sera The maximal amount of antibody fixed onto the Rh positive cells corresponded to 1/16–1/32 MAD in the anti sheep cell system and to 1/4–1/8 MAD in the anti AB system The results suggest that the limited number of available Rh receptors might be a sufficient explanation of the incompleteness of the anti-Rh antibodies

The heavily sensitized (with 1/2 MAD) sheep red cells and group AB cells could be used 'diagnostically' for the demonstration of the RF, emphasizing the significance of the amount of sensitizing γ globulin fixed on the cell surface However, the behavior of the rare "diagnostic" anti Rh sera could not be explained on this quantitative basis

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The National Bacteriological Laboratory, Stockholm Sweden

A NON-SPECIFIC INHIBITOR TO VACCINIA HAEMAGGLUTINATION IN POST MORTEM HUMAN SERA

By

J ÅKE ESPMARK and BIRGITTA MAGNUSSON

Received 26 v 64

The vaccinia haemagglutination-inhibition (HI) test is known to be a valuable and convenient diagnostic aid, at least in the initial phase of a smallpox outbreak (3, 5, 8) Being extensively used during the smallpox outbreak in Stockholm in 1963, the usefulness of the HI test could be confirmed, on the whole, also by us (6)

During the latter outbreak a certain alarm was evoked by the incidental finding of a high HI titre in a serum sample drawn post mortem from an unvaccinated man, who had died suddenly from an acute illness with fever, purpura and an atypical rash

It was soon discovered, however, that this high HI titre was due to a non specific inhibitor, and consequently a large number of post mortem sera were investigated

The occurrence of such inhibitors is reported in this short communication An account is also given of the possibility of removing or inactivating the inhibitors by pre-treatment of sera with different agents

Non specific inhibitors to vaccinia haemagglutinin have been found previously in such materials as umbilical cord blood and ascites fluid (1) *Szathmary et al* described similar inhibitors in various pathological body fluids such as pleural exudate, fluid from hydrocele testis and ovarial cysts etc (11) *Szathmary* also found that kaolin absorption, and to a certain extent the action of trypsin, could remove these non specific inhibitors

MATERIAL AND METHODS

Vaccinia Haemagglutinating Antigen (HA Antigen)

Bottle cultures of HFp 2 [illegible] cent calf serum were infect[illegible] chick embryo passage Aft[illegible] vanced the liquid was cha[illegible] After 2 days of further inc[illegible] [illegible] centrifuged and the supernatant saved as the HA antigen

Sera

Forty five post mortem blood samples were obtained at autopsies performed 9 to 83 hours after death All samples were taken at the same hospital and reached the

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within 9-12 hours reach a high titre which subsequently does not increase appreciably.

TABLE 1

Distribution of HI Titres to Vaccinia Haemagglutinin in 45 Post Mortem Sera Titrated without Treatment and after Absorption with Kaolin

	Numbers of sera with the respective HI titres								Total number of sera
	< 10	10	20	40	80	160	320	640	
Untreated sera	2	3	2	8	10	11	4	5	45
Sera treated with kaolin	41	2	1*		1†				45

* Fresh vaccinia pock
† Vaccinated 1 month before death

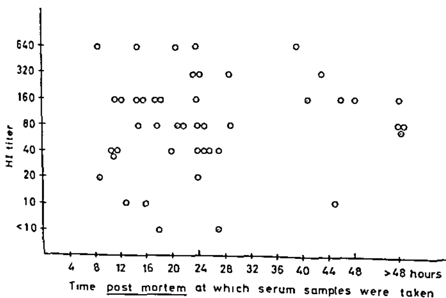


Fig 1

Inhibitory titres to vaccinia haemagglutinin in 45 post mortem sera drawn at different times after death

Further Attempts to Remove the Non-Specific HI Titres

The non-specific inhibition titres were not significantly affected by the routine heat inactivation The action of four different methods of serum pre-treatment was studied in a comparative test The agents tested were trypsin, chloroform, periodate and kaolin, which are known to remove effectively non specific inhibitors in other HI systems (*e g* myxovirus and arbo-virus HI tests) Fifteen post mortem sera and five positive and five negative sera were treated on the same day and then run in a simultaneous HI test

laboratory within 4 hours Serum was taken off immediately and then stored frozen at —25° C until used [1]

Positive normal control sera were obtained from verified smallpox cases A number of prevaccination sera served at negative normal controls

Serum Treatment

All sera even those termed 'untreated in the sequel were heated at 60° C for 20 minutes and then absorbed with one 10th volume of rooster erythrocytes

The following methods were tested for their ability to remove or destroy the non specific inhibitors present in post mortem sera

Kaolin treatment (2) One volume of serum was mixed with four volumes of a '25 per cent' suspension of acid washed kaolin (Fisher Scientific Co Fair Lawn N Y) After 20 minutes at room temperature under occasional shaking the mixture was centrifuged at 2500 r p m for 15 minutes The clear supernatant was used as serum diluted 1 5

Chloroform treatment (9, 12) To 1 volume of serum were added 4 volumes of chloroform (analytic grade), the mixture was shaken for 2 minutes and then left for 30 minutes at room temperature The top layer (extracted serum) separated by an opaque disc from the bottom chloroform layer, was pipetted off and centrifuged for 20 minutes at 5000 r p m The supernatant was considered as undiluted serum

Periodate treatment (7) Three volumes of a 0 011-M solution of potassium periodate (KIO_4) in saline were added to one volume of serum and the mixture was left at room temperature for 20 minutes The oxidation process was interrupted by addition of one volume of a 3 per cent glycerol solution

Trypsin treatment (10) One volume of a 0 8 per cent trypsin solution (Trypsin 1–300, NBC) in phosphate buffered saline pH 8 2 was mixed with 2 volumes of serum The mixture was kept in a water bath at 56° C for 30 minutes

Haemagglutination Inhibition (HI) Test

The HI test was performed in tubes with two fold serum dilutions, starting from 1 10 and a constant amount of antigen (4 HA units per tube) A 0 5 per cent suspension of sensitive rooster erythrocytes was added 10 minutes after the antigen the tubes were shaken and kept for 1 hour at 37° C, after which bottom patterns were read The HI titre expressed as the reciprocal of initial serum dilution was determined by the last tube showing complete inhibition of the haemagglutination

RESULTS

Level of Non-Specific Titres and the Time of their Occurrence

Almost all of the 45 post mortem sera had appreciable HI titres when tested without prior treatment High titres, *i e* 80 or more, were found in 30 out of the 45 sera It is evident that such titres would be highly suspect during a smallpox outbreak if their non specific nature were not recognized However, treatment of sera with kaolin reduced the titres drastically The distributions of HI titres in untreated and kaolin treated sera are shown in Table 1 The two significant titres remaining after kaolin absorption were probably specific as judged from vaccination histories

Sera had been drawn at different times, *i e* from 9 to 83 hours after death As seen in Fig 1, the correlation is poor between the height of the non-specific titres and the time after death at which blood samples were taken Evidently the inhibitors develop soon after death and,

[1] The authors are much indebted to Dr A *Hoberg* Södersjukhuset Stockholm for valuable help in collecting sera and certain clinical data

treatment The chemical nature of the inhibitor was not further investigated

The titre of the inhibitor in haemagglutination-inhibition tests with vaccinia antigen was of the same magnitude as the specific titres found among smallpox cases The recognition of the non-specific inhibitor is of practical importance in epidemic episodes where specimens from obscure cases of sudden death may be subjected to laboratory investigation

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TABLE 2

The Effect of Treating post mortem Sera with Various Agents to Remove Non Specific Inhibitory Activity against Vaccinia Haemagglutinin

		Vaccinia HI titres of sera untreated and after treatment with various agents				
		Untreated	Trypsin	*Chloroform*	Periodate	Kaolin
Post mortem sera	1	80	80	10	< 10	< 10
	2	40	80	10	< 10	< 10
	3	80	320	20	< 10	< 10
	4	80	160	40	10	< 10
	5	640	160	40	20	20
	6	160	160	20	10	< 10
	7	320	160	10	10	< 10
	8	640	320	80	160	80
	9	320	160	10	< 10	< 10
	10	640	640	20	< 10	< 10
	11	640	640	< 10	< 10	< 10
	12	640	320	40	< 10	< 10
	13	320	320	10	< 10	< 10
	14	40	10	< 10	< 10	< 10
	15	40	40	10	10	< 10
Variola conv al sera	V 1	320	160	160	320	80
	V 2	640	640	320	640	160
	V 3	640	640	640	640	320
	V 4	640	640	640	640	640
	V 5	160	160	160	320	160
Neg human sera	N 1	< 10	< 10	10	< 10	< 10
	N 2	< 10	< 10	< 10	< 10	< 10
	N 3	< 10	< 10	< 10	< 10	< 10
	N 4	< 10	< 10	< 10	< 10	< 10
	N 5	< 10	< 10	< 10	< 10	< 10

The effect of the various treatments is seen in Table 2 The negative control sera were unaffected by the treatments The specific titres of *positive sera were somewhat reduced by the kaolin absorption* but not significantly changed by the other agents

The HI titres of the post mortem sera could be reduced or completely removed by periodate and kaolin with approximately the same efficiency, whereas chloroform exhibited a marked but less complete effect There was no unequivocal effect of the trypsin treatment

CONCLUSION AND SUMMARY

The majority of post mortem sera were found to contain inhibitors to high titres against vaccinia haemagglutinin The inhibitory activity was demonstrable as early as 9 hours after death It could be removed by treatment with kaolin and periodate and partly removed through extraction with chloroform, but was virtually unaffected by trypsin

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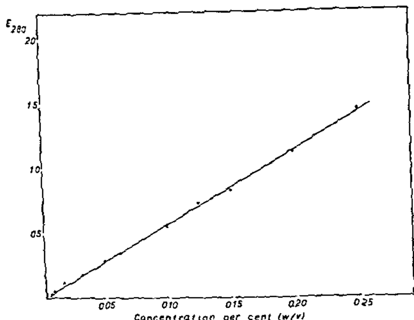


Fig 1

Calibration curve showing the extinction at 280 mμ in a spectrophotometer of different concentrations of β lipoprotein (obtained by dilution of the standard β lipoprotein solution)

Standard β Lipoprotein Solution

A 2 per cent solution (w v) of human serum β lipoprotein prepared from whole [illegible] 63 (Op Nr 22263), was [illegible] ingwerke AG Marburg [illegible] of less than 1 063 The [illegible] standard solution was measured in a Beckman DU spectrophotometer using a 1 cm cell and a calibration curve for β lipoprotein was drawn (Fig 1)

Chromatographic Isolation of Human Serum β Lipoprotein

Hydroxylapatite was prepared as described by *Tiselius Hjertén & Levin* (1956) Hydroxylapatite columns with a height of 150 mm and a diameter of 12 mm [illegible] used [illegible]

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Acta path et microbiol scandinav 62 600–612 1964

The University Institute of Forensic Medicine Rikshospitalet, Oslo Norway
and the University Institute of Human Genetics Marburg Germany

IMMUNOCHEMICAL STUDIES OF THE Lp(a) FACTOR

By

KÅRE BERG

Received 30 vii 64

The Lp system was described by *Berg* (1963) In gel diffusion tests (*Ouchterlony* 1958) absorbed rabbit immune sera distinguished genetic types of human serum β-lipoprotein The β lipoprotein factor, demonstrated in this way, was called the Lp(a) factor Individuals possessing this factor in their serum were said to be of type Lp(a+), those lacking it of type Lp(a—) The genetically determined Lp types are independent of previously known serum type and blood group systems (*Berg & Mohr* 1963, *Mohr & Berg* 1963, *Berg* 1964 a)

Antisera can be obtained by intravenous immunization of rabbits with either isolated β lipoprotein or whole human serum of type Lp(a+) (*Berg* 1965 a)

The reaction obtained with Lp(a+) human sera was weaker when the sera had been stored for several months at —25° C (*Berg* 1963) *It was therefore obviously necessary to study the stability of the Lp(a)* factor

In the present paper, further data on the characterisation and the stability of the Lp(a) factor are presented The chromatographic method of *Hjerten* (1959), which the present author uses for purification of human β-lipoprotein, is also considered in this paper The isolated β-lipoprotein, obtained by this technique, is useful for immunization of rabbits and further immunochemical study of the Lp(a) factor

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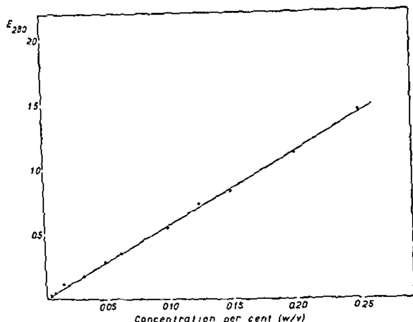


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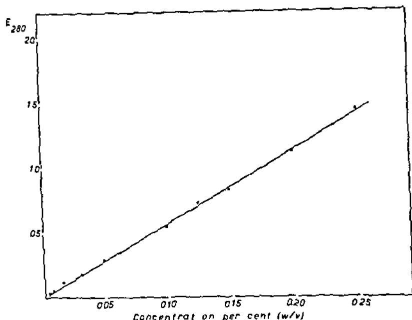


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Sera from 35 patients, suffering from different conditions requiring multiple blood transfusions were used in one experiment The patients had each received at least 5 blood transfusions The sera were obtained from different clinical departments [1]

All blood samples were obtained by venepuncture treated as described elsewhere (*Berg* 1963), and the sera were stored at —25° C until used

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Double Diffusion Tests in Agar Gel

The tests were performed either in Petri dishes filled with 24 ml, or on glass slides covered with 2.5 ml agar. The gel contained 1 per cent (w/v) agar, 0.85 per cent (w/v) NaCl, 10 per cent (v/v) 1/15 M sodium phosphate buffer of pH 7.0 and 1/10000 (w/v) merthiolate. Dishes and slides were equally good for the Lp tests (Berg 1964 b).

Six peripheral wells and one central well were cut in the agar, the diameter of each well being 4 mm, and the distances between neighbouring wells in all cases being 5 mm. The slides and dishes were kept in a moist chamber at 37° C. Readings of precipitates were made against a dark background by oblique illumination from below after 1, 2, 3 and 7 days. Visible precipitate was recorded as positive reaction, no visible precipitate as negative reaction.

Immunoelectrophoresis

Immunoelectrophoresis was performed as described by *Grabar & Williams* (1953) with the micromodification of *Scheidegger* (1955).

Staining

The precipitates in the gel diffusion tests were stained according to the method of *Uriel* (1960) using either Oil Red O as a lipid stain or Amido Black 10 B as a protein stain (both from G. T. Gurr Ltd., London). The presence of esterase activity in precipitates was demonstrated as described by *Uriel* (1961), using β-naphthyl acetate (L. Light & Co. Ltd., Colnbrook, England) and Diazo Blue B (G. Gurr Ltd., London).

Photographic Registrations

The dishes and the slides were photographed in the moist, unstained condition in indirect illumination. A Leica camera and 35 mm Agepe Agfa film were used.

EXPERIMENTS AND RESULTS

Characterization of the Lp(a) Factor as a Component of β Lipoprotein of Lp(a+) Sera

The Lp(a) factor can, at present, only be studied by means of specific anti-Lp(a)-serum. Lp(a+) and Lp(a−) human sera (5 μl of each) were submitted to immunoelectrophoresis, and the antiserum trough was filled with a specific anti Lp(a)-serum (100 μl). Lp(a+) human sera gave one single precipitate, while sera of type Lp(a−) gave no precipitate. The precipitate corresponded to that of β-lipoprotein of human serum, with respect to the length of electrophoretic migration, the distance from the antibody trough, and the shape of the precipitation arc (Fig. 2).

It was found necessary to use as much as 5 μl Lp(a+) human serum to obtain visible precipitates. This might indicate that the Lp(a) factor is a comparatively small part of the total β-lipoprotein. Different dilutions of human serum were therefore tested against unabsorbed rabbit immune serum. A human serum was diluted 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160 with a 0.85 per cent saline solution. 5 μl of each dilution was submitted to immunoelectrophoresis. An unabsorbed sample of the immune serum used in the experiment described above was diluted with an equal part of 0.85 per cent saline solution. This gave the same dilution effect as the absorption of the same antiserum for use as anti-Lp(a) serum. Each of the antibody troughs was filled with 100 μl of

of rabbits the different β lipoprotein batches were adjusted to give an extinction as close to this value as possible

The optical density was usually measured in 1/10 dilutions of the β lipoprotein fractions and the concentration of β lipoprotein was read from the calibration curve There is probably no major objection to the use of a *solution of lipoproteins* of density less than 1 063 prepared in the ultracentrifuge as a standard for the β lipoprotein fraction obtained by the chromatographic method as *Cramer* (1962) has found that the latter contains lipoproteins with densities from 1 063 to less than 1 006 No further quantitative studies of the β lipoprotein were within the scope of the present study

Preparation of Lipoproteins in the Ultracentrifuge

Ultracentrifugation was carried out in the 40 rotor of a Spinco model L preparative ultracentrifuge[1] at 105 000 G for 24 hours From each of the sera of 2 Lp(a+) and 2 Lp(a—) individuals lipoproteins of densities < 1 006 < 1 019 and < 1 063 respectively were prepared as described by *Havel Eder & Bragdon* (1955)

Rabbit Anti Human Serum Antiserum

Anti Humanserum (Behringwerke AG Marburg Op Nr 470 I) was used as antiserum in the immunoelectrophoretic tests of serum protein fractions

Specific Rabbit Anti Human β Lipoprotein Antiserum

Anti α_2 Lipoproteinserum[2] (Behringwerke AG Marburg Op Nr 320 D) was used

Anti Lp(a) Sera

Sera from rabbit No 1 absorbed 2 3 from No 3 absorbed 2 5 and from No 52 absorbed 2 3 with Lp(a—) human serum were used

Sera from rabbit No 3 and No 52 were also used in the unabsorbed state for some of the experiments.

Dialysis Bags

Cellophane Casings from Visking Co Chicago Ill were used

Concentration of Chromatographic Fractions

Concentration was performed by dialysis against Polyethylene glycol 20 000 (Hoechst) as described by *Kohn* (1959)

Petri Dishes

ANUMBRA dishes with a diameter of 7 cm were used

Glass Slides

Washed and polished 5 × 5 cm glass slides (Menzel Gläser) were used

Agar

Rheinagar (Behringwerke AG Marburg Op Nr 18 141 142 147 148 150) and Bacto Agar (Difco Laboratories Detroit 1 Mich Control No 452925) were used The Bacto Agar was washed and stored as described by *Hirschfeld* (1960) before use For the Lp tests the first agar is just as good as the second and the only reason for using different sorts of agar was that one was more easily available than the other at the time of the investigations

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1 I am indebted to Dr *P Bjornstad* Institute of Clinical Biochemistry University of Oslo for permission to use the ultracentrifuge

2 The designation of this antiserum refers to the behaviour of the β lipoprotein in agar gel immunoelectrophoresis

Double Diffusion Tests in Agar Gel

The tests were performed either in Petri dishes filled with 24 ml, or on glass slides covered with 2.5 ml agar. The gel contained 1 per cent (w/v) agar, 0.85 per cent (w/v) NaCl, 10 per cent (v/v) 1/15 M sodium phosphate buffer of pH 7.0 and 1:10 000 (w/v) merthiolate. Dishes and slides were equally good for the Lp tests (*Berg* 1964 b).

Six peripheral wells and one central well were cut in the agar, the diameter of each well being 4 mm and the distances between neighbouring wells in all cases being 5 mm. The slides and dishes were kept in a moist chamber at 37° C. Readings of precipitates were made against a dark background by oblique illumination from below after 1, 2, 3 and 7 days. Visible precipitate was recorded as positive reaction, no visible precipitate as negative reaction.

Immunoelectrophoresis

Immunoelectrophoresis was performed as described by *Grabar & Williams* (1953) with the micromodification of *Scheidegger* (1955).

Staining

The precipitates in the gel diffusion tests of *Uriel* (1960) using either Oil Red O as a protein stain (both from G. T. Gurr Ltd., Lonc in precipitates was demonstrated as describ acetate (L. Light & Co. Ltd. Colnbrook, Engl London).

Photographic Registrations

The dishes and the slides were photographed in the moist unstained condition in indirect illumination. A Leica camera and 35 mm Agepe Agfa film were used.

EXPERIMENTS AND RESULTS

Characterization of the Lp(a) Factor as a Component of β Lipoprotein of Lp(a+) Sera

The Lp(a) factor can, at present, only be studied by means of specific anti Lp(a)-serum. Lp(a+) and Lp(a—) human sera (5 μl of each) were submitted to immunoelectrophoresis, and the antiserum trough was filled with a specific anti Lp(a)-serum (100 μl). Lp(a+) human sera gave one single precipitate, while sera of type Lp(a—) gave no precipitate. The precipitate corresponded to that of β-lipoprotein of human serum, with respect to the length of electrophoretic migration, the distance from the antibody trough, and the shape of the precipitation arc (Fig. 2).

It was found necessary to use as much as 5 μl Lp(a+) human serum to obtain visible precipitates. This might indicate that the Lp(a) factor is a comparatively small part of the total β-lipoprotein. Different dilutions of human serum were therefore tested against unabsorbed rabbit immune serum. A human serum was diluted 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160 with a 0.85 per cent saline solution. 5 μl of each dilution was submitted to immunoelectrophoresis. An unabsorbed sample of the immune serum used in the experiment described above was diluted with an equal part of 0.85 per cent saline solution. This gave the same dilution effect as the absorption of the same antiserum for use as anti-Lp(a)-serum. Each of the antibody troughs was filled with 100 μl of

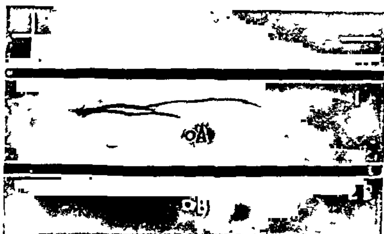


Fig 2

Immunoelectrophoretic characterisation of the Lp(a) factor The reagents were

A Human serum of type Lp(a+)
B Human serum of type Lp(a—)
1 Rabbit-anti human serum-antiserum
2 Anti Lp(a) serum

A precipitate corresponding to the β lipoprotein of the Lp(a+) serum is found against the anti-Lp(a) serum whereas no precipitate can be seen between the Lp(a—) serum and the antiserum

this diluted, unabsorbed rabbit immune serum A distinct precipitate was observed corresponding to the β-lipoprotein of the serum portions diluted 1/40 or less, whereas no precipitate could be seen where the human serum had been diluted 1/80 or more

In double diffusion tests, β-lipoprotein fractions, as well as fractions from the other steps of the chromatography, were tested against anti Lp(a)-serum A precipitate developed between the antiserum and β-lipoprotein from Lp(a+) individuals when the concentration of the fraction was 0 20 0 25 per cent (w v) or higher No precipitate was formed between the antiserum and fractions from the other steps of the chromatography, or between the antiserum and β lipoprotein of type Lp(a—), even when the fraction had a considerably higher concentration than mentioned above

Lipoproteins of densities $< 1\ 006$, $< 1\ 019$ and $< 1\ 063$, prepared in the ultracentrifuge from Lp(a+) and Lp(a—) individuals, were tested against anti-Lp(a)-serum A precipitate was found only against the lipoprotein of density $< 1\ 063$ from Lp(a+) individuals From this it was concluded that the Lp(a) factor belongs to the 1 019–1 063 density class of human serum lipoproteins

Lp(a+) human serum was dialysed against equal parts of a 0 85 per cent saline solution for 24 hours The serum and the dialysate were thereafter tested against anti Lp(a)-serum, and it was found that the Lp(a+) serum still reacted with the antiserum, whereas no reaction was observed between the antiserum and the dialysate It was concluded that the Lp(a) factor is not removed from whole human serum by

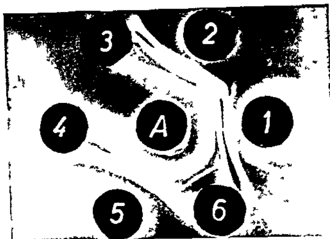


Fig 3

Spur formation between a Lp(a+) and a Lp(a—) human serum (wells 1 and 2) The reagents were

A Unabsorbed rabbit immune serum
1 Human serum of type Lp(a+)
2 and 5 Human serum of type Lp(a—)
3 and 4 0 85 per cent saline solution
6 Anti Lp(a) serum

dialysis Several of the β lipoprotein batches were also tested before the dialysis described in the section dealing with chromatographic isolation of this protein The test results were always identical to those obtained after dialysis, indicating that this procedure had no major damaging effect on the Lp(a) factor

All precipitates between anti Lp(a) serum and Lp(a+) human serum, or between the antiserum and solutions of isolated Lp(a+) β lipoprotein, could be stained by protein and lipid dyes, and the presence of esterase could be demonstrated The Lp(a) factor is therefore a lipoprotein possessing esterase activity

Lp(a+) and Lp(a—) human sera were tested in double diffusion experiments against unabsorbed rabbit immune sera When the immune sera contained anti Lp(a) antibody, a spur could usually be seen between the precipitates formed by Lp(a+) and Lp(a—) human sera, indicating that the Lp(a+) serum possessed at least one antigenic component more than the Lp(a—) sera (Fig 3)

In some experiments, an additional precipitate near the well containing Lp(a+) serum developed instead of a spur (Fig 4)

The additional line of precipitation showed a reaction of identity with the precipitate obtained with anti Lp(a) serum This experiment demonstrated that the Lp(a) factor is a separate antigenic component, different from the rest of the β lipoprotein The additional precipitate observed in some experiments, might even indicate that the Lp(a) factor is found in a separate group of β lipoprotein molecules

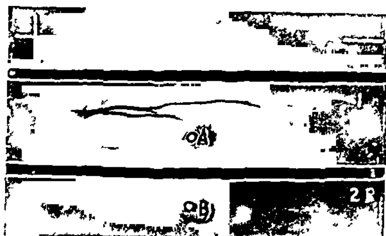


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Lipoproteins of densities < 1.006, < 1.019 and < 1.063, prepared in the ultracentrifuge from Lp(a+) and Lp(a—) individuals, were tested against anti-Lp(a)-serum A precipitate was found only against the lipoprotein of density < 1.063 from Lp(a+) individuals From this it was concluded that the Lp(a) factor belongs to the 1.019–1.063 density class of human serum lipoproteins

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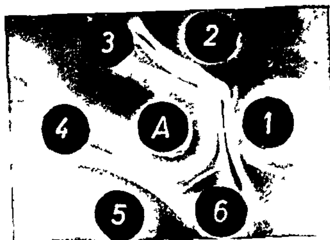


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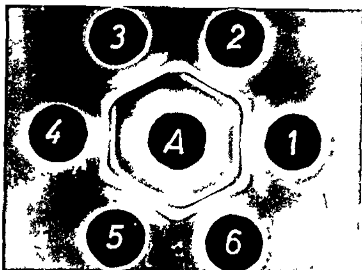


Fig 4
Precipitation pattern between unabsorbed rabbit immune serum (well A) and Lp(a+) (wells 1, 3 and 5) and Lp(a—) (wells 2 4 and 6) human sera Note additional precipitation line adjacent to wells 1, 3 and 5 The serum of well 5 had been stored for 9 months all other sera were fresh

Qualitative Constancy of the Lp(a) Factor in Serum of Lp(a+) Individuals

Sera from the panel donors, from several different bleedings in the course of a 2-year period, were tested for the Lp(a) factor

From 21 *additional individuals, blood* was drawn 3 *times* at *intervals* of one week, and the sera submitted to Lp tests

From 8 individuals, three blood samples were drawn on one day the first in the morning when the individuals were fasting, the second 4 hours after breakfast and the third 3 hours after a high fat meal

In all these experiments, serum from the same individual always gave the same reaction when tested against anti Lp(a)-serum It was concluded that the Lp(a) factor is constantly present in the serum of Lp(a+) individuals, and that the result of the Lp test of one individual's serum is not influenced by fasting or high fat intake

The Effect of Repeated Freezing and Thawing on the Lp(a) Factor

Fifty fresh Lp(a+) human sera were subjected to repeated freezing and thawing The sera were thawed once a day and put back into the deepfreeze at —25° C as soon as they had all thawed completely They were tested against anti-Lp(a) serum after different numbers of thawings, and the reactions recorded After repeated thawings some of the sera gave very weak reactions Even the precipitates that were close to the limit of visibility were recorded as positive reactions The results were also recorded in this way when testing the effect of storage under different conditions, described in the following sections Thus negative



Fig 5

The effect of repeated freezings and thawings on the Lp(a) factor 50 Lp(a+) human sera were tested against anti Lp(a) serum after different numbers of thawings

reaction always means that no precipitate could be seen, whereas sera giving very weak and doubtful precipitates are found among those recorded as reacting positively after different lengths of storage time The results of the Lp tests after different numbers of thawings are shown in Fig 5

It will be seen that after the tenth thawing one of the Lp(a+) sera did not react visibly with the antiserum any more, and by later tests, the number of sera still reacting positively decreased steadily When the sera had been thawed 100 times, only 5 sera still reacted positively

It was thus evident that the process of freezing and thawing is damaging to the Lp(a) factor

The Effect of Storage at —25° C on the Lp(a) Factor

50 Lp(a+) sera from freshly drawn blood samples were stored at —25° C for 12 months and tested again for the Lp(a) factor Only 34

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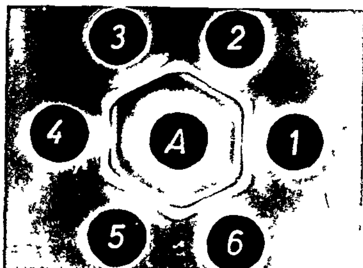


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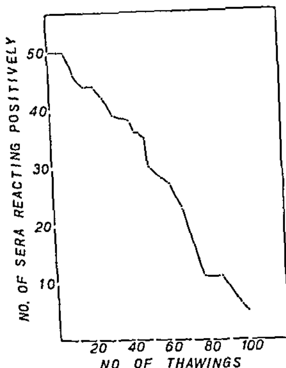


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The Effect of Storage at —25° C on the Lp(a) Factor

50 Lp(a+) sera from freshly drawn blood samples were stored at —25° C for 12 months and tested again for the Lp(a) factor Only 34 (68 per cent) of the sera gave a visible precipitate against the antiserum after this period of storage These sera had not been thawed during the period between the first and the second testing 35 Lp(a—) sera treated

in the same way were also tested The results of the first and second tests were identical

In addition, sera which had been stored at —25° C for different lengths of time were tested These sera had not been submitted to Lp typing in the fresh state The sera were divided into groups according to the period of storage, and the number of Lp(a+) and Lp(a—) sera in each group was compared by means of "fourfold" tables (*Hill* 1961, p 172) with the numbers found in a material of freshly drawn sera from the same population (Marburg, Germany) The results are shown in Table 1, where the χ^2 for each comparison is included

TABLE 1

The Results of Lp Tests of Sera Stored for Different Length of Time at —25° C

Storage time months	Number of sera			χ^2	(1 D F)
	Total	Lp(a+)	Lp(a—)		
0	301	98 (32.56%)	203 (67.44%)	–	
1– 3	95	30 (31.58%)	65 (68.42%)	0.0027	(0.95 < P < 0.98)
3– 9	92	20 (21.74%)	72 (78.26%)	3.4276	(0.05 < P < 0.10)
9–15	88	12 (13.64%)	76 (86.36%)	11.1057	(P < 0.001)
15 21	100	11 (11.00%)	89 (89.00%)	16.5525	(P < 0.001)

The χ^2 was calculated as recommended by *Hill* (1961, p 172), by comparing each group of stored sera with the fresh sera (storage time 0)

In the groups of sera which had been stored for 9–15 and 15 21 months, the number of Lp(a+) sera was significantly lower than in the material of fresh sera The most probable explanation is that a number of sera from Lp(a+) individuals had lost their precipitating ability during the period of storage

130 human sera which had been stored at —25° C for 6 years were tested None of these sera reacted positively with anti-Lp(a)-serum, probably because the Lp(a) factor had lost its ability to form a visible precipitate

12 of these sera were tested against anti-β-lipoprotein-serum All of the sera gave distinct precipitates, indicating that at least a part of the β-lipoprotein still possessed its ability to react with an immune serum

Samples of the 20 *panel sera were tested after storage at* —25° C for periods of different lengths When the Lp(a+) sera had been stored for about 5–6 months, the precipitates formed with anti Lp(a)-serum were usually weaker than those obtained with fresh sera After 8 9 months of storage, some of the samples of Lp(a+) sera had lost their precipitating ability against specific antiserum, but samples still reacting after 2 years of storage were also found

On two occasions it was observed that samples of Lp(a+) sera had lost their ability to form visible precipitates after 5 months of storage at —25° C

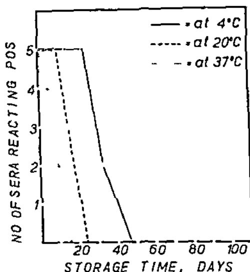


Fig 6

The effect of storage at different temperatures without any added preservative on the Lp (a) factor Samples of 5 Lp(a+) sera were tested against anti Lp(a) serum after different lengths of storage time

It was concluded that the results of the Lp testing will probably provide correct results only when the sera have been stored at —25° C for less than about 5 months

No preservative had been added to the sera described in this and the preceding sections

The Effect of Storage at Different other Temperatures and of the Addition of Merthiolate on the Lp(a) Factor

Samples of 5 Lp(a+) and 5 Lp(a—) human sera were stored at 4°, 20° and at 37° C Samples of the same sera to which merthiolate in a final concentration of 1/10 000 (w/v) had been added, were stored under identical conditions The sera were tested for the Lp(a) factor several times during the period of storage, and the results recorded In Fig 6 the number of Lp(a+) sera still reacting positively at different times is shown for each of the three temperatures

The results found when merthiolate had been added to the sera are shown in Fig 7

Storage at 37° C gave identical results, whether merthiolate was present or not whereas merthiolate was found to have a preserving effect on the Lp(a) factor at the two lower temperatures

No precipitate was observed between anti-Lp(a)-serum and any of the Lp(a—) sera in these tests

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In addition, sera which had been stored at —25° C for different lengths of time were tested. These sera had not been submitted to Lp typing in the fresh state. The sera were divided into groups according to the period of storage, and the number of Lp(a+) and Lp(a—) sera in each group was compared by means of "fourfold" tables (*Hill* 1961, p. 172) with the numbers found in a material of freshly drawn sera from the same population (Marburg, Germany). The results are shown in Table 1, where the χ^2 for each comparison is included.

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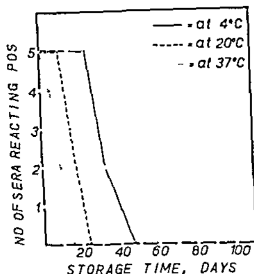


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The effect of storage at different temperatures, without any added preservative on the Lp(a) factor Samples of 5 Lp(a+) sera were tested against anti Lp(a)-serum after different lengths of storage time

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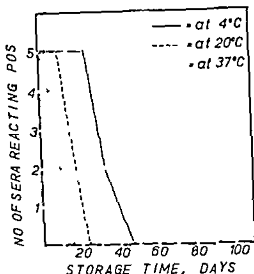


Fig 6

The effect of storage at different temperatures without any added preservative on the Lp(a) factor Samples of 5 Lp(a+) sera were tested against anti Lp(a) serum after different lengths of storage time

It was concluded that the results of the Lp testing will probably provide correct results only when the sera have been stored at —25° C for less than about 5 months

No preservative had been added to the sera described in this and the preceding sections

The Effect of Storage at Different other Temperatures and of the Addition of Merthiolate on the Lp(a) Factor

Samples of 5 Lp(a+) and 5 Lp(a—) human sera were stored at 4°, 20° and at 37 C Samples of the same sera to which merthiolate in a final concentration of 1/10 000 (w/v) had been added were stored under identical conditions The sera were tested for the Lp(a) factor several times during the period of storage, and the results recorded In Fig 6 the number of Lp(a+) sera still reacting positively at different times is shown for each of the three temperatures

The results found when merthiolate had been added to the sera are shown in Fig 7

Storage at 37° C gave identical results, whether merthiolate was present or not whereas merthiolate was found to have a preserving effect on the Lp(a) factor at the two lower temperatures

No precipitate was observed between anti Lp(a) serum and any of the Lp(a—) sera in these tests

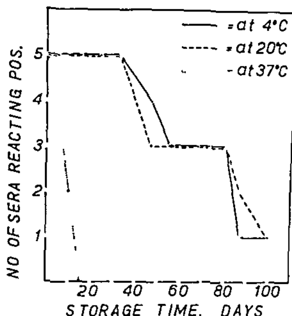


Fig 7

The effect of storage at different temperatures on the Lp(a) factor when merthiolate had been added to the sera as a preservative Samples of 5 Lp(a+) sera were tested against anti Lp(a) serum after different lengths of storage time

The Effect of Heat on the Lp(a) Factor

Samples of two Lp(a+) sera and one Lp(a—) serum were heated for 15 minutes at 40°, 50°, 60° and 70° C respectively After heating, the samples were tested against anti-Lp(a)-serum and anti-β-lipoprotein-serum The samples of the Lp(a+) sera which had been heated to 70° C did not react visibly with anti Lp(a)-serum, whereas those heated to lower temperatures formed distinct precipitates None of the samples of the Lp(a—) serum formed a precipitate when tested against anti Lp(a)-serum

All heated serum samples were precipitated by anti-β lipoprotein-serum

Studies on the Antigenicity of the Lp(a) Factor

To investigate whether the Lp(a) factor might have led to the production of isoantibodies in patients who had received multiple blood transfusions, sera of 35 such patients, 5 of type Lp(a+), 30 of type Lp(a—), were tested All the patients had received more than 5 and some of them more than 40 transfusions

The tests were performed on agar slides against the 20 panel sera No anti-Lp(a) antibody was demonstrable in these sera The finding of isoprecipitins with other specificities in some of these sera will be reported elsewhere

It was concluded that isoimmunization with the Lp(a) factor, if it

occurs at all, is probably rare. Studies on the heteroantigenic properties of the Lp(a) factor are reported in another paper (*Berg* 1965 a).

DISCUSSION

The results presented provide evidence that the Lp(a) factor is a separate antigenic component within human serum β-lipoprotein. It is also possible that the Lp(a) factor is located on a separate group of lipoprotein molecules, different from the rest of the β-lipoprotein molecules. This finding is consistent with earlier reports of immunological heterogeneity of the β-lipoproteins (*Gitlin* 1953, *Aladjem, Lieberman & Gofman* 1957, *Aladjem & Campbell* 1957), and with the detection of *Berg* (1964 a) that the Lp(a) and Ag(a) factors are located on different β-lipoprotein molecules.

A true, antigenic difference between Lp(a+) and Lp(a—) human sera is also indicated by the striking difference in the effect of absorption with Lp(a+) and Lp(a −) human sera (see *Berg* 1963, Table 1). Further, no anti-Lp(a) antibody could be demonstrated in the sera of rabbits immunized with human serum or β-lipoprotein of type Lp(a—) (Berg 1965 a). Finally, the qualitative constancy of the Lp(a) factor in the serum of Lp(a+) individuals supports the assumption of a true antigenic difference. A direct comparison between the amount of β-lipoprotein and the Lp type has, however, not been performed.

It seems reasonable to presume that the Lp(a) factor constitutes a comparatively small part of the total β-lipoprotein.

The instability of the Lp(a) factor is consistent with present knowledge of the degradation of serum lipoproteins (*Lindgren & Nichols* 1960, *Briner, Riddle & Cornwell* 1959). The present data may indicate that the Lp(a) factor is more easily degraded than the rest of the β-lipoproteins.

The protecting effect of the addition of merthiolate to the human sera stored at 4° and 20° C indicates that bacterial action may play an important part in the degradation of the Lp(a) factor. This is consistent with the findings made by *Briner, Riddle & Cornwell* (1959) with lipoprotein fractions.

From the data obtained on the stability of the Lp(a) factor, it is evident that human sera should be tested for the Lp(a) factor in as fresh a state as possible.

SUMMARY AND CONCLUSIONS

1. The Lp(a) factor is a separate, antigenic component perhaps even a separate protein, within the β-lipoproteins of Lp(a+) individuals. It probably constitutes a comparatively small part of the total β-lipoprotein.

2. The Lp(a) factor belongs to the lipoproteins of density class 1.019–1.063.

3 Studies on the stability of the Lp(a) factor are presented From these experiments, it is evident that the factor is degraded by storage, even in the frozen state, and by freezings and thawings The necessity of using fresh human sera for the Lp typing is stressed

4 Isoimmunization with the Lp(a) factor, in patients receiving multiple blood transfusions, has so far not been observed

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STUDIES ON THE REACTION BETWEEN Lp(a+) HUMAN SERA AND ANTI-Lp(a)-SERA FROM RABBITS

By

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Received 30 xii 64

The study of the genetically determined Lp(a) factor of human serum β lipoprotein (*Berg* 1963), is at present possible only by means of rabbit immune sera The absorbed immune sera, anti-Lp(a)-sera, react in agar gel double diffusion tests (*Ouchterlony* 1958) only with human sera possessing the Lp(a) factor (Lp(a+) human sera), and not with those lacking it (Lp(a—) human sera) When equal parts of undiluted Lp(a+) human serum and anti-Lp(a)-serum are allowed to diffuse against each other at 37° C in agar gel of standard composition (*Berg* 1963), a distinct precipitate develops after 12–14 hours (see *Berg* 1963, Fig 4) It is located one third to half the way from the well containing human serum to that filled with antiserum The presence of protein, lipid and esterase activity in the precipitate can be demonstrated by different staining techniques In the present paper the precipitate will be referred to as the Lp(a) precipitate or the Lp(a) reaction

The method to be used as a standard for typing within the Lp system, should be reliable and practical Several factors known to influence antigen antibody reactions must be considered to secure optimal conditions for Lp typing

The purpose of the present work was to study the effect of different factors on the Lp(a) reaction The results of the experiments performed serve as a guide for the choice of a standard method for Lp testing

MATERIALS AND METHODS

Human Sera

Human sera from the standard panel and from other healthy donors (*Berg* 1964 b) were used The sera were used within a few weeks of storage at —25° C

Hemolysate from Lp(a—) Individual

[illegible] minutes The supernatant was pipetted off and used for experiments later described

Anti Lp(a) Sera

Sera from rabbit No 1 absorbed 2 3, from No 3 absorbed 2 5 and from No 52 absorbed 2 3 with *Lp(a—) human serum* were used (see *Berg* 1965 a)

Petri Dishes

ANUMBRA dishes, with a diameter of 7 cm were used

Glass Slides

Washed and polished 5 × 5 cm glass slides (Menzel Gläser) were used

Agar

Rheinagar (Behringwerke AG, Marburg Op -Nr 18, 141, 142 147 148 150) and Bacto Agar (Difco Laboratories Detroit 1, Mich Control No 452925) were used The Bacto Agar was washed as described by *Hirschfeld* (1960) before use

Agar Cutter

The wells in the agar gel were punched with a precision cutter with a removable cover and an outlet to a suction pump With this instrument the agar could be removed from the wells during the process of punching The cutter was made to order (manufacturer Wilhelm Vogel Giessen Germany)

Double Diffusion Tests

The tests were performed either in Petri dishes filled with 24 ml or on 5 × 5 cm glass slides covered with 2 5 ml agar The standard composition of the gel and the design of the wells for the reagents were as previously described (*Berg* 1964 b) In the dishes each well was usually filled with 60 µl on the slides with 15 µl reagent When nothing else is particularly stated slides and dishes were kept in a moist chamber at 37° C Readings of precipitates, recording of reactions and photographic registrations were made as described by *Berg* (1964 b)

The reliability of the standard method for Lp testing was demonstrated by the *fact that the panel sera which were tested numerous times in different experiments* always gave the same test results In addition 212 sera were tested twice on 5 × 5 cm slides in Rheinagar of the standard composition The results of the two experiments were identical

Staining

The agar slides were stained according to the methods of *Uriel* (1960 1961) as previously described (*Berg* 1964 b)

EXPERIMENTS AND RESULTS

The Effect of Type of Agar and Thickness of Gel Layer on the Lp(a) Reaction

One per cent (w/v) agar gel of the standard composition was prepared in the following ways

(a) in Petri dishes, each containing 24 ml Bacto Agar giving a layer 6 mm thick
(b) on 5 × 5 cm glass slides each covered with 2 5 ml Bacto Agar giving a layer 1 mm thick
(c) on 5 × 5 cm glass slides, each covered with 2 5 ml Rheinagar giving a layer 1 mm thick

100 normal human sera were tested blindly against anti-Lp(a)-serum on each of the two types of agar slides and in the dishes The 100 sera gave the same test results against anti-Lp(a)-serum in the three sets of experiment It was concluded that all the three modifications provide reliable results of the Lp tests The slide technique has the advantage of being more economical with the reagents

The Effect of the Concentration of Agar in Gel on the Lp(a) Reaction

The experiments described in this and the following sections were all made in duplicate, and the dishes were observed after 2, 3, 4, 5, 6, 10, 12, 14 24, 36 and 48 hours, and after 3 and 7 days

Petri dishes were prepared containing 0 5, 1, 2, 3 and 5 per cent (w/v) Bacto Agar (24 ml) respectively, all other constituents of the gel being as described in the standard procedure In these dishes, anti-Lp(a)-serum in the central well was allowed to diffuse against Lp(a+) and Lp(a—) human sera from the peripheral wells

In the dishes where the agar concentration was 0 5–1 5 per cent, a precipitate between Lp(a+) human serum and anti Lp(a)-serum was observed 12 hours after the start of the experiment When the agar concentration was 2 per cent, a very weak precipitate could be seen after 14 hours, and a distinct precipitate after 24 hours In the dishes containing 3 per cent agar the Lp precipitate was observed after 36 hours, and in the dishes with 5 per cent agar, a trace of a precipitate could be seen after 48 hours or more Once the Lp precipitates had developed, they did not disappear No precipitates occurred between anti-Lp(a)-serum and Lp(a—) human serum or saline solution

In the dishes with 0 5 per cent agar, circular precipitates occurred around all wells which had been filled with human sera or immune sera At this low concentration as well as at the two highest concentrations of agar the gel is difficult to handle As already mentioned, the precipitates occur later with 2 and 3 per cent agar in the gel than at a concentration of 1 0 or 1 5 per cent

It was concluded that a gel containing 1 0 or 1 5 per cent agar should be used for the Lp tests The 1 0 per cent gel has the advantage of being more economical and is therefore preferred

The Effect of pH in the Agar Gel on the Lp(a) Reaction

In agar dishes of otherwise standard composition, the pH was varied by addition of buffers with different pH values The volume of buffer was as given in the standard description Barbital buffer with an ionic strength of 0 1 was used to obtain a pH of 8 6 For all other pH values, 1 15 M phosphate buffers were used Dishes with the following pH values were prepared 5 0 6 0 7 0, 8 0 and 8 6 At pH 7 0, 8 0 and 8 6 the Lp(a) precipitates could be seen after 12 hours, at pH 5 0 and 6 0 weak precipitates were observed after 14 hours Circular precipitates

developed around the wells in the dishes containing gel of pH 5.0 and 6.0 and to a lesser degree at pH 8.6. It was therefore concluded that an agar medium with a pH of 7.0–8.0 should be used for the Lp tests.

The Effect of Concentration of NaCl in the Agar Gel on the Lp(a) Reaction

Dishes with the following concentrations of NaCl in the agar gel were prepared: 0, 0.45, 0.9, 2.0, 3.0, 5.0 and 10.0 per cent (w/v). All other constituents of the agar medium were as described in the standard procedure.

The time required for the development of Lp(a) precipitates at the different concentrations of NaCl, is shown in Table 1.

TABLE 1

Reaction Pattern between Lp(a+) Human serum and Anti Lp(a)-Serum in Agar Gel Double Diffusion Tests at Different Concentrations of NaCl in the Agar Medium

Concentration of NaCl in agar gel per cent (w/v)	Reaction time hours (time after start of experiments)									
	2	5	10	12	14	21	36	48	72	168
0	—	—	—	?	?	?	?	?	?	?
0.45	—			?	?	?	+	+	+	+
0.9		—	—	+	+	+	+	+	+	+
2.0		—	—	—	—		+	+	+	+
3.0	—	—	—	—	—		—	(+)	+	+
5.0		—	—			—	—	(+)	+	+
10.0	—	—	—	—	—		—	—	(+)	+

\+ = distinct precipitate
(+) = weak precipitate
— = no visible precipitate
? = reaction impossible to read because of circular precipitates around the wells

When the NaCl concentration was 2.0 per cent or higher the time necessary for the development of Lp(a) precipitates was considerably increased. The precipitates were also distinct at these higher NaCl concentrations.

In the dishes containing agar with no or 0.45 per cent NaCl, large, dense, circular precipitates developed around the wells. These concentric precipitates made it impossible to read the Lp reactions in agar containing no NaCl. In the gel with 0.45 per cent NaCl, the Lp reactions could only be clearly read after 36 hours.

It was concluded that a NaCl concentration of 0.9 per cent (w/v) in the agar gel should be used for the Lp tests.

The Effect of Temperature on the Lp(a) Reaction

All experiments so far described were performed at 37° C. To test the effect of temperature on the Lp(a) reaction, Petri dishes containing

gel of the standard composition were kept at 4, 20 and 37° C. In addition to the readings already mentioned, these dishes were also observed after 16, 18, 20 and 22 hours. Distinct precipitates between Lp(a+) human sera and anti-Lp(a)-sera were observed after 12 hours at 37° C, after 14 hours at 20° C, and after 22 hours at 4° C. No other differences were observed. The precipitates did not disappear once they had developed.

The Lp tests can obviously be performed at any of the three temperatures.

The results of the Lp tests are obtained earlier at 37° C than at lower temperatures, and incubation at 37° C is therefore preferred.

The Effect of Different Ratios of Reagents on the Lp(a) Reaction

Different amounts of undiluted human serum and anti-Lp(a)-serum were tested on 5 × 5 cm agar slides. When more than 15 μl of a reagent was used, the well was refilled as soon as the previously introduced serum had diffused into the surrounding agar gel.

Fifteen μl Lp(a+) human serum in the central well was tested against the following volumes of anti-Lp(a)-serum in the peripheral wells: 5, 10, 15, 20, 40 and 60 μl. As a control, the same experiment was also performed with Lp(a—) human serum.

In another experiment, 15 μl anti-Lp(a)-serum in the central well was tested against the following amounts of Lp(a+) human serum in the peripheral wells: 5, 10, 15, 20, 40 and 60 μl. This experiment was also performed with Lp(a—) human serum.

In both experiments the most favourable reactions with respect to localization, density and time of appearance of the Lp(a) precipitate were found where about equal amounts of antiserum and Lp(a+) human serum reacted with each other. When excess of antiserum was used, the Lp(a) precipitate was located close to the well containing human serum and was very difficult to read. When, however, 60 μl Lp(a+) human serum was tested against 15 μl anti-Lp(a) serum, the precipitate was still located comparatively far away from the well containing antiserum, and was easy to read. When the smallest amount (5 μl) of either Lp(a+) human serum or anti-Lp(a) serum was used, the Lp(a) precipitate was very weak, and required a long time of reaction to develop.

No precipitate between Lp(a—) human serum and anti-Lp(a)-serum was observed in these experiments.

It was concluded that equal parts of human sera, and the anti-Lp(a)-serum employed in the described experiments, should be used for testing for the Lp(a) factor.

Experiments similar to those dealt with in this section should be performed with each new anti-Lp(a)-serum to ascertain which ratio between antiserum and human serum should be used in the testing for

developed around the wells in the dishes containing gel of pH 5.0 and 6.0 and to a lesser degree at pH 8.6. It was therefore concluded that an agar medium with a pH of 7.0–8.0 should be used for the Lp tests.

The Effect of Concentration of NaCl in the Agar Gel on the Lp(a) Reaction

Dishes with the following concentrations of NaCl in the agar gel were prepared: 0, 0.45, 0.9, 2.0, 3.0, 5.0 and 10.0 per cent (w/v). All other constituents of the agar medium were as described in the standard procedure.

The time required for the development of Lp(a) precipitates at the different concentrations of NaCl, is shown in Table 1.

TABLE 1

Reaction Pattern between Lp(a+) Human serum and Anti Lp(a) Serum in Agar Gel Double Diffusion Tests at Different Concentrations of NaCl in the Agar Medium

Concentration of NaCl in agar gel per cent (w/v)	Reaction time hours (time after start of experiments)									
	2	5	10	12	14	21	30	48	72	108
0	—	—	—	?	?	?	?	?	?	?
0.45	—	–		?	?	?	+	+	+	+
0.9	—	—	—	+	+	+	+	+	+	+
2.0	—	—	—	—	—		+	+	+	+
3.0	—	—	—		—		—	(+)	+	+
5.0	—	—	—	—	—		—	(+)	+	+
10.0	—	—	—	—	—	—	—	—	(+)	+

+ = distinct precipitate
(+) = weak precipitate
— = no visible precipitate
? = reaction impossible to read because of circular precipitates around the wells

When the NaCl concentration was 2.0 per cent or higher the time necessary for the development of Lp(a) precipitates was considerably increased. The precipitates were also distinct at these higher NaCl concentrations.

In the dishes containing agar with no or 0.45 per cent NaCl large, dense, circular precipitates developed around the wells. These concentric precipitates made it impossible to read the Lp reactions in agar containing no NaCl. In the gel with 0.45 per cent NaCl, the Lp reactions could only be clearly read after 36 hours.

It was concluded that a NaCl concentration of 0.9 per cent (w/v) in the agar gel should be used for the Lp tests.

The Effect of Temperature on the Lp(a) Reaction

All experiments so far described were performed at 37° C. To test the effect of temperature on the Lp(a) reaction, Petri dishes containing

The Effect of Complement on the Lp(a) Reaction

Fresh panel sera were tested against an anti Lp(a) serum Samples of the panel sera and the anti Lp(a) serum were kept at 56° C for 30 minutes in order to inactivate complement Samples of panel sera treated in this way were thereafter tested against untreated as well as inactivated anti Lp(a) serum In addition untreated samples of panel sera were tested against inactivated anti Lp(a) serum In all these experiments the test results were identical to those obtained in the first test when only untreated reagents were used It was concluded that the presence of active complement is not necessary for the development of the Lp(a) precipitate

A Special Technique for the Testing of Sera from Hemolysed Blood Samples

When sera from hemolysed blood samples are tested dense circular precipitates develop around the wells These precipitates make the reading of the Lp(a) reaction difficult or impossible A procedure separating the β lipoprotein from the disturbing components of hemolysed blood cells prior to diffusion against anti Lp(a) serum was therefore developed The amounts of sera were the same as those of the standard technique on glass slides The following method combining electrophoretic separation of β lipoprotein from the constituents of the hemolysate at pH 7 0 with diffusion against anti Lp(a) serum was applied

5 × 5 cm slides were covered with 2 5 ml 1 per cent (w/v) Bacto Agar in a 0 05 M sodium phosphate buffer of pH 7 0 On these slides 3 wells were cut in the gel 2 near one end of the slide the third more centrally as shown in Fig 1

Each well had a diameter of 4 mm the distance from the circumference of the central well to the other wells was 10 mm and the distance between the circumferences of the latter two wells 8 mm

The two wells near the end of the slide were each filled with 15 μl human serum to be tested and the electrophoresis was started The electrode vessels contained 0 05 M sodium phosphate buffer with pH 7 0 and the filter paper (Whatman No 3) connecting electrode buffer compartments with the agar slides were soaked in this buffer before use The negative pole was at the end near the human sera The electrophoresis was carried on for 2½ hours at 100 V and 35 mA During the electrophoresis β lipoprotein moved towards the positive pole and was thereby separated from the main components of the hemolysate which moved towards the negative pole After electrophoresis 15 μl anti Lp(a) serum was introduced into the central well the slides were kept in a moist chamber at 37° C and the reactions read after 1 2 and 3 days (no precipitates took more than 2 days to develop) The human sera were classified as Lp(a+) or Lp(a−) depending upon whether a line

the Lp(a) factor. So far, all the anti-Lp(a)-sera used for routine testin have given optimal reactions when equal parts of antiserum and human serum were used. These results were also expected, as the absorption ratios for the typing reagents were selected from experiments with equal parts of human serum and absorbed immune serum

The Effect of Dilution of the Reagents on the Lp(a) Reaction

Anti-Lp(a)-serum was diluted with a 0 85 per cent saline solution The following dilutions were used 1/1 (undiluted), 1/2, 1/4, 1/8, 1/16 1/32, 1/64 Five Lp(a+) human sera and one Lp(a—) serum were diluted in the same way Corresponding dilutions of antiserum and human sera were tested against each other in double diffusion experiments on glass slides The agar slides were observed after 1, 2, 3 and 7 days, and thereafter weekly for 4 weeks When undiluted (1/1 dilution) reagents were tested against each other, distinct precipitates were observed against all Lp(a+) human sera after 1 day On the slides where reagents diluted 1/2 were tested, the Lp(a) reactions required 2 days to develop After 3 days, a precipitate had been formed by 4 of the 5 Lp(a+) human sera in the experiment where the reagents were diluted 1/4, and after 7 days the last Lp(a+) human serum also reacted positively No precipitate was observed when the reagents were diluted 1/8 or more The precipitates formed by Lp(a+) human sera did not disappear once they had developed No precipitate was observed between antiserum and the Lp(a—) human serum in any of the experiments

It was concluded that undiluted reagents should be used for the Lp tests

The Effect of Merthiolate on the Lp(a) Reaction

Agar dishes of the standard composition apart from not containing merthiolate were prepared, and Lp(a+) and Lp(a) human sera were tested against anti-Lp(a)-serum The test results were identical to those obtained in gel containing merthiolate It was thus obvious, that the merthiolate added to the gel does not interfere with the Lp(a) reaction

Merthiolate was added to a sample of anti Lp(a)-serum and to samples of each of the 20 panel sera to a final concentration of 1/10 000 (w/v). In standard agar dishes, anti Lp(a)-serum containing merthiolate was tested against the panel sera with and without merthiolate, and anti-Lp(a)-serum *without merthiolate was tested against panel* sera containing merthiolate In these experiments the test results were always identical to those obtained with the panel sera and anti-Lp(a)-serum, when none of the reagents contained merthiolate *It was* concluded that merthiolate added to the reagents in a concentration of 1/10 000 (w/v) had no damaging effect on the Lp(a) reaction

or in whole human serum This precipitate therefore does not represent any source of error in the experiments employing whole human serum With the standard composition of the gel, and at reaction temperature of 37° C, the mentioned non-immune precipitate near a well containing isolated β lipoprotein occurs about 3 hours after the start of the experiment, and disappears 7–9 hours later It has therefore usually disappeared when the Lp(a) precipitate develops These reaction conditions are therefore also useful for the study of the Lp type of isolated β lipoprotein When the concentration of NaCl in the gel is 2 per cent or higher, the development of the described non-immune precipitate is prevented

Optimal conditions for the Lp(a) reaction were found when equal amounts of undiluted antiserum and human serum were used, but the precipitate also developed after some dilution of the reagents, and when different proportions of the reacting sera were used Some dilution of the sera by admixture of saline from the pipettes should therefore not affect the results of the tests It is further reason to believe that variations in the amount of β-lipoprotein between individual human sera do not influence the test results Experiments with different amounts of sera and with diluted sera should, however, be performed with all new antisera to ascertain that the results are similar to those described in the present paper

The test technique gives reliable results when fresh human sera are used During storage, the Lp(a) factor deteriorates, even at —25° C (*Berg* 1964 b) The Lp(a) factor is better preserved when merthiolate is added This phenomenon, as well as the presence of the factor in the serum of different bleedings of the same individual, are dealt with in another paper (*Berg* 1964 b)

Samples of venous blood should probably be preferred as some dilution by admixture of tissue fluid may take place when capillary blood is drawn This is consistent with the finding of some "weak reactions" in samples from capillary blood (*Berg & Mohr* 1963) In the absence of hemolysis, however, these weak reactions are also well above the limit of visibility In paternity cases, only serum samples from capillary blood from the children are usually available It is the experience of the present author that these blood samples often are hemolysed It is then necessary to test the sera by the technique designed for this purpose as the circular precipitates caused by these sera may mask the Lp(a) precipitate

The loss of reactivity of human sera on storage could indicate that the presence of active complement was essential for the Lp(a) reaction The experiments prove, however, that active complement is not essential for the reaction The change in reactivity of sera during storage is probably due to lability of the β lipoprotein molecule

Some of the experiments in the present study sustain the theory that the reaction between anti-Lp(a)-serum and Lp(a+) human serum is

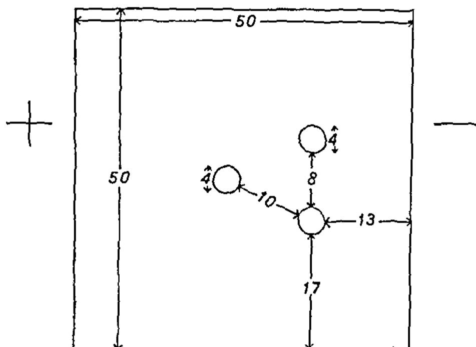


Fig 1

Arrangement of the wells on the agar slide used for the special test method designed for sera from hemolysed blood samples (see text) The diameter of each well is 4 mm All measures are in millimetres

of precipitation could be observed or not (see *Berg* & *Mohr* 1963, Fig 2) To test the reliability of this method, 60 human sera already tested with the standard technique, were tested blindly by means of the method described in this section For each of these sera, 1 drop of hemolysate from a Lp(a—) individual was added to 9 drops of serum, before testing with the present technique

The test results obtained with the two methods were identical It was therefore concluded that the procedure described can be used for the Lp testing of sera contaminated by hemolysate

DISCUSSION

The present experiments have shown that the Lp(a) reaction manifests itself over a wide variety of reaction conditions It can be read at several values of pH and at several concentrations of NaCl and agar in the gel The use of 5 × 5 cm glass slides instead of dishes has the advantage of being more economical The composition of the gel described in the standard procedure, and a reaction temperature of 37° C, are preferred Under these conditions it is easy to distinguish the Lp(a) reaction from a non-immune precipitate between isolated human serum β-lipoprotein and albumin observed by *Berg* (1964 a) This precipitate was only observed when β-lipoprotein in the isolated state was used, whereas albumin could be present either in the isolated state

or in whole human serum This precipitate therefore does not represent any source of error in the experiments employing whole human serum With the standard composition of the gel, and at reaction temperature of 37° C, the mentioned non-immune precipitate near a well containing isolated β lipoprotein occurs about 3 hours after the start of the experiment, and disappears 7 9 hours later It has therefore usually disappeared when the Lp(a) precipitate develops These reaction conditions are therefore also useful for the study of the Lp type of isolated β lipoprotein When the concentration of NaCl in the gel is 2 per cent or higher, the development of the described non immune precipitate is prevented

Optimal conditions for the Lp(a) reaction were found when equal amounts of undiluted antiserum and human serum were used, but the precipitate also developed after some dilution of the reagents, and when different proportions of the reacting sera were used Some dilution of the sera by admixture of saline from the pipettes should therefore not affect the results of the tests It is further reason to believe that variations in the amount of β-lipoprotein between individual human sera do not influence the test results Experiments with different amounts of sera and with diluted sera should, however, be performed with all new antisera to ascertain that the results are similar to those described in the present paper

The test technique gives reliable results when fresh human sera are used During storage, the Lp(a) factor deteriorates, even at —25° C (*Berg* 1964 b) The Lp(a) factor is better preserved when merthiolate is added This phenomenon, as well as the presence of the factor in the serum of different bleedings of the same individual, are dealt with in another paper (*Berg* 1964 b)

Samples of venous blood should probably be preferred as some dilution by admixture of tissue fluid may take place when capillary blood is drawn This is consistent with the finding of some "weak reactions in samples from capillary blood (*Berg* & *Mohr* 1963) In the absence of hemolysis, however, these weak reactions are also well above the limit of visibility In paternity cases, only serum samples from capillary blood from the children are usually available It is the experience of the present author that these blood samples often are hemolysed It is then necessary to test the sera by the technique designed for this purpose, as the circular precipitates caused by these sera may mask the Lp(a) precipitate

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The loss of reactivity of human sera on storage could indicate that the presence of active complement was essential for the Lp(a) reaction The experiments prove however, that active complement is not essential for the reaction The change in reactivity of sera during storage is probably due to lability of the β lipoprotein molecule

Some of the experiments in the present study sustain the theory that the reaction between anti-Lp(a)-serum and Lp(a+) human serum is

a specific immunological reaction and not due to subtle quantitative differences between human sera. The absence of precipitate between Lp(a—) human serum and anti-Lp(a)-serum as well when excess of either human serum or antiserum was used is especially significant.

Further evidence that the reacting factor of rabbit immune serum is a true, specific antibody (*Berg* 1965 b), and that the Lp(a) factor is a specific antigenic component of human β-lipoprotein (*Berg* 1964 b), is presented in other papers.

SUMMARY AND CONCLUSIONS

The reaction between Lp(a+) human serum and anti-Lp(a)-serum is a true, specific, *immune precipitation*. The presence of active complement is not necessary.

The effect on the Lp(a) reaction of the composition of the gel, the reaction temperature and the amount of reacting sera has been studied. Based on these experiments, a standard test procedure is proposed. It is stressed that the human sera to be tested should be fresh.

A special test method for sera from hemolysed blood samples is presented.

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TRANSACTIONS
OF THE PATHOLOGICAL SOCIETY OF NORWAY

Meeting February 13, 1964

Oslo, Norway

Schult Haudt S D CURRENT ASPECTS ON THE BIOCHEMISTRY OF COLLAGEN

Chevremont & Frederic MITOCHONDRIA

A picture shown and commented by *O H Iversen*

Meeting April 16, 1964

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Scarff R W AN INFORMATION ON ICSP

Fagerhol M K THE DETERMINATION OF BLOOD GROUPS IN BLOOD DONORS AT NORWEGIAN HOSPITALS

The frequency of errors in blood group determinations in the years 1961 63 has been determined for 52 Norwegian hospitals In 37 hospitals using standard techniques the ABO grouping failed in 34 of 7321 groupings i e 0 46 per cent The Rh typing failed in 93 of 6559 typings i e 1 51 per cent In 15 hospitals using Eldon cards ABO grouping failed in 6 of 829 groupings i e 0 72 per cent The Rh typing failed in 37 of 854 typings i e 4 33 per cent It is recommended that the ABO and Rh grouping of all new blood donors should be controlled in a blood group laboratory and that the hospitals should perform ABO and Rh typing prior to this control to detect and eliminate systematic errors in the local blood grouping procedure Only well established techniques which permit adequate controls should be used

Ioe K THE PERCENTAGE INCIDENCE OF SEROPOSITIVE SYPHILIS IN PREGNANT WOMEN IN NORWAY 1958 1963

The incidence of infectious syphilis has increased in many parts of the world during the last five years in Norway only a small rise during the last year (1963) The frequency of seropositive syphilis in the population may also give some information regarding this problem In Norway all women are controlled with serological syphilis tests in the pregnancy In the Sero diagnostic department State Institute of Public Health Oslo blood samples from 173 176 pregnant women were examined by serological syphilis tests during the five year period from May 1,

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1958 to May 1 1963 Sera giving positive standard tests were also tested with the TPI test to exclude false positive reactors

A total of 654 pregnant women had positive standard tests of syphilis of which 183 were TPI negative The average incidence of seropositive syphilis thus being 0 27 per cent (471 pregnant women) during the whole five year period

The annual rate has decreased during the same period from 0 38 per cent in the first year (1/5 58 1/5 59) to 0 17 per cent in the last year The rate for the last year is the lowest rate ever observed in this laboratory The frequency of seropositive syphilis in the five year period is reduced in all age groups also in the youngest groups

The investigation indicates that in this five year period 1958 1963 syphilis has been reduced among the female population in Norway

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